AUS DEM LEHRSTUHL
FÜR NEUROLOGIE
PROF. DR. ULRICH BOGDAHN
DER MEDIZINISCHEN FAKULTÄT
DER UNIVERSITÄT REGENSBURG

ADULT NEUROGENESIS IN TRANSGENIC ANIMAL MODELS OF DYT1 PRIMARY TORSION DYSTONIA

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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Dekan:	Prof. Dr. Bernhard Weber
1. Berichterstatter:	PD Dr. Beate Winner
2. Berichterstatter:	Prof. Dr. Andreas Luchner
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Contents

A. Summary (english, german)

ADULT NEUROGENESIS IN TRANSGENIC ANIMAL MODELS OF DYT1 PRIMARY TORSION DYSTONIA

Primary torsion dystonia is a hereditary movement disorder characterized by generalized, sustained, involuntary muscle contractions with an underlying three-base-pair deletion in the DYT1 gene coding for torsinA. The precise role of torsinA and the effect of its mutated form remain unclear to date, but its functions have been linked to chaperones, secretory cellular mechanisms, dopaminergic metabolism and to plasticity and synaptogenesis. **Adult neurogenesis** is the generation of new neurons throughout adulthood restricted to the two neurogenic zones of the adult mammalian brain, the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles. It is affected by many acute and degenerative diseases of the central nervous system and might be responsible for some of their symptoms. Moreover, it is regulated by striatal dopamine levels and provides a form of cellular neural plasticity.

We investigated the effect of torsinA on adult neurogenesis in two previously described transgenic mouse models of DYT1-related torsion dystonia, one group overexpressing human wildtype torsinA and the other human mutant torsinA. Animals underwent a standard BrdU injection paradigm and were examined immunohistochemically 28 days later. We found that transgenic torsinA was not expressed within neural precursor cells in both groups. Proliferation within the neurogenic zones and survival of the newly generated cells, as detected by the numbers of PCNA- and BrdU-positive cells, respectively, did not differ from control animals. In addition, there were no signs of striatal neurogenesis or neuronal migration into the striatum.

Hence, the transgene has neither an endogenous nor an exogenous influence on adult neural stem and progenitor cells in these *in vivo* models and adult neurogenesis is not altered in this model. However, more studies are needed to further rule out a possible function of torsinA in embryonic neurogenesis.

DIE ADULTE NEUROGENESE IN TRANSGENEN TIERMODELLEN DER PRIMÄREN DYT1-TORSIONSDYSTONIE

Die **primäre Torsionsdystonie** ist eine erbliche Bewegungsstörung, bei der es aufgrund einer 3-Basenpaar-Deletion im DYT1-Gen zu generalisierten, unwillkürlichen und anhaltenden Muskelkontraktionen kommt. Die exakte Rolle des Genprodukts TorsinA und dessen mutierter Form ist noch immer unbekannt, aber dessen Funktion wurde mit Chaperonen, sekretorischen Zellmechanismen, dem Dopaminstoffwechsel und mit Plastizität und Synaptogenese in Verbindung gebracht. Die **adulte Neurogenese** ist die Neubildung von Neuronen während des Erwachsenenalters und ist physiologischerweise begrenzt auf zwei neurogene Zonen des adulten Säugergehirns: den Gyrus dentatus des Hippokampus und die Subventrikulärzone der Seitenventrikel. Die adulte Neurogenese ist bei vielen akuten und degenerativen Erkrankungen des zentralen Nervensystems verändert und trägt möglicherweise zu einem Teil der Symptome bei. Außerdem wird sie durch die Dopaminspiegel moduliert und stellt eine Form zellulärer neuraler Plastizität dar.

In dieser Arbeit wurde der Effekt von TorsinA auf die adulte Neurogenese in zwei bereits charakterisierten transgenen Mausmodellen von DYT1-Torsionsdystonie untersucht. In der einen Gruppe war die Wildtyp-Form von TorsinA überexprimiert, in der anderen die mutierte Form. Die Tiere erhielten wiederholte BrdU-Injektionen und wurden 28 Tage später immunhistochemisch untersucht. In keiner der beiden Gruppen war transgenes TorsinA in neuralen Vorläuferzellen exprimiert. Die Proliferation in den neurogenen Zonen und das Überleben der neugebildeten Zellen wurden durch Zählung PCNA- und BrdU-positiver Zellen ermittelt. Diese zeigten keine Unterschiede zwischen transgenen und Kontroll-Gruppen. Zudem zeigte sich kein Hinweis auf striatale Neurogenese oder neuronale Wanderung in das Striatum.

Daher hat das Transgen in diesen *in-vivo*-Modellen weder einen endogenen noch einen exogenen Einfluss auf adulte neurale Stamm- und Progenitorzellen und führt auch zu keiner Veränderung der adulten Neurogenese. Dennoch sind weitere Untersuchungen nötig, um eine Auswirkung von TorsinA insbesondere auf die embryonale Neurogenese auszuschließen.

B. Introduction

1. Primary torsion dystonia

***History*:** Dystonias are a group of movement disorders characterized by sustained involuntary contractions of one or more groups of muscles resulting in abnormal body postures. A first clear description of dystonia dates back to 1911 when the neurologist Hermann Oppenheim observed a variety of hypertonic muscles while most of the other muscles were hypotonic at the same time in several young boys (Oppenheim 1911). He termed this disease "dystonia musculorum deformans" (Fahn 1988; Grundmann 2005).

Clinical features and classifications: Dystonias comprise a clinically and genetically heterogenous group of movement disorders characterized by sustained muscle contractions affecting one or more sites of the body, frequently causing twisting and repetitive movements or abnormal postures due to central nervous dysfunction (Fahn et al. 1998). The term "dystonia" can serve as description for the clinical symptom of these sustained muscle contractions. In addition, it can refer to the clinical syndrome when dystonia is part of another underlying neurological disorder (i.e. secondary or symptomatic dystonia) or refer to an own entity (i.e. primary or idiopathic dystonia) when the symptom dystonia is the primary and sole abnormality attributable to the condition.

Thus, primary dystonia patients show no other phenotypic abnormality than dystonic movements and for these, no known cause apart from genetic mutations can be found (Tarsy and Simon 2006). Secondary dystonias, on the other hand, have a spectrum of different known causes including neurodegenerative diseases like Parkinson's disease (PD) or Huntington's disease (HD) and including drug-induced dystonia as a side-effect of antipsychotics. In a third category, dystonia-plus syndromes have been distinguished from secondary dystonias because they show other neurological signs in addition to dystonia, e.g. parkinsonism in dopa-responsive dystonia.

<table>
<tr><th>Dystonia as a syndrome</th><th colspan="2">Etiology</th><th>Age at onset</th><th>Topographic Distribution</th><th>Topographic Progression</th><th>Prevalence per 100.000</th></tr>
<tr><td rowspan="5">as an own entity</td><td rowspan="5">primary</td><td rowspan="2">hereditary (DYT1, 2, 4, 6, 7, 13, 16)</td><td>infantile</td><td>generalized</td><td rowspan="2">frequent / fast</td><td rowspan="4">3</td></tr>
<tr><td rowspan="4">juvenile</td><td rowspan="2">hemidystonia</td></tr>
<tr><td rowspan="3">sporadic</td><td>multifocal</td></tr>
<tr><td rowspan="3">adult</td><td>segmental</td><td rowspan="3">rare / slow</td></tr>
<tr><td>focal</td><td>30</td></tr>
<tr><td rowspan="4">as part of another disease</td><td rowspan="2">dystonia plus</td><td>myoclonus</td><td colspan="3">sporadic and hereditary (DYT11, 15) forms</td><td rowspan="4">7</td></tr>
<tr><td>dystonia parkinson syndromes</td><td colspan="3">sporadic and hereditary (DYT3, 5, 12) forms</td></tr>
<tr><td>secondary (sympto-matic)</td><td colspan="4">e.g. caused by neurodegenerative disorders, drugs, ischemia, injury, psychogenic</td></tr>
<tr><td>paroxys-mal</td><td colspan="4">sporadic and hereditary (DYT8, 9, 10) forms</td></tr>
</table>

Table1. Different ways of classifying dystonia syndromes and how they frequently correlate, combined from Fahn et al. (1998), Bressman (2004), Tarsy and Simon (2006) and Schmidt et al. (2008).

Cases are classified according to the topographic distribution of symptoms, age of onset and familial occurrence (de Carvalho Aguiar and Ozelius 2002). Late-onset dystonia typically develops after the age of 25, shows no family history and is most likely to remain localized as focal dystonia or as segmental dystonia. The most common types of focal dystonia are writer's cramp (Fig.1A), blepharospasm and torticollis (Fig.1B). Patients with an early-onset before the age of 25 often show a family history of dystonia and are most likely to develop generalized dystonia (primary torsion dystonia, PTD, Fig.1C). In most patients, movements become worse during action or concentration and can be improved by relaxation or by a sensory

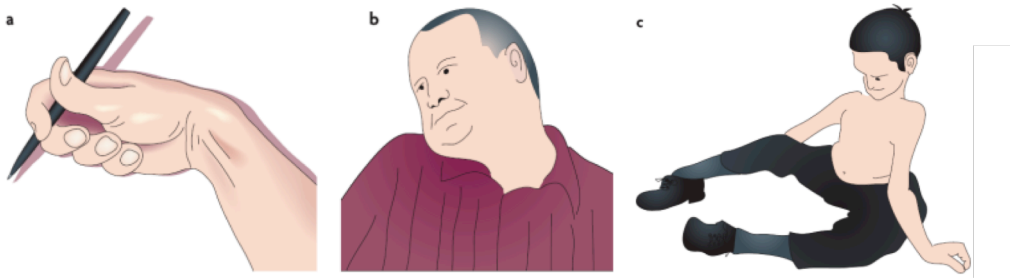


Figure 1. **Different types of dystonia**, adapted from Breakefield et al. (2008): (A) Writer's cramp is a form of task-specific adult-onset dystonia with disabling contractions of hand, finger and forearm muscles during handwriting. (B) Torticollis is characterized by sustained or intermittent contractions of the neck muscles forcing the head into an abnormal tilt. (C) Symptoms of childhood/adolescent-onset forms of dystonia like PTD start in one leg or arm and progress into other parts of the body leading to abnormal postures.

trick (geste antagoniste).

Dystonia represents the third most common movement disorder. Primary focal dystonias occur in 30 of 100.000 people which makes them about ten times more common than primary generalized dystonias. However, a large number of cases remains un- or misdiagnosed (Greene et al. 1995; Defazio et al. 2004).

Regarding medical treatment, some patients partially benefit from anticholinergic, GABAergic or dopaminergic pharmacotherapy, but the treatment of choice for focal dystonias are repeated local injections of botulinum neurotoxin. For more severe or generalized cases, pallidal deep-brain stimulation is currently investigated with predominantly positive influences on the symptoms (Schrader et al. 2009). However, these approaches are symptomatic and no cure has been found so far because underlying disease mechanisms have not been fully understood.

Monogenic types of dystonia: As seen in table 2, 16 gene loci have been described up to now as causes of monogenic forms of dystonia. Distinct clinical features have been described for each mutation (Klein 2008). Many of the monogenic dystonias are inherited in an autosomal-dominant manner with limited penetrance suggesting that a 'second hit' (genetic or environmental) is needed to trigger the disease. The reported gene loci have different prevalences with rare ones that have been found in some families only pointing to a founder mutation to others with a big spread in the population indicating a relatively high rate of de-novo mutations (Klein and Ozelius 2002). The DYT1 mutation is present in about 70% of all hereditary PTD cases while the majority of primary adult-onset dystonias appears to be sporadic.

Name	**Dystonia type**	**OMIM**	**Inheritance**	**Locus**	**Gene product**
DYT1	Early-onset generalized torsion dystonia	128100	AD	9q34	TorsinA
DYT2	Autosomal recessive torsion dystonia	224500	AR	Unknown	Unknown
DYT3	Dystonia-parkinsonism ("lubag")	314250	XR	Xq13.1	Disease-specific changes in DYT3 region
DYT4	'Non-DYT1' dystonia (whispering dystonia)	128101	AD	Unknown	Unknown
GCH1	Dopa-responsive dystonia	128230	AD	14q22.1-2	GTP-cyclohydrolase1
TH	Segawa syndrome	191290	AR	11p15.5	Tyrosine hydroxylase
DYT6	Adolescent-onset dystonia of mixed type	602629	AD	8p21-22	Unknown
DYT7	Adult-onset focal dystonia	602624	AD	18p	Unknown
DYT8	Paroxysmal non-kinesigenic dyskinesia	118800	AD	2q33-25	Myofibrillogenesis regulator 1

Name	Dystonia type	OMIM	Inheritance	Locus	Gene product
DYT9	Paroxysmal choreoathetosis with episodic ataxia and spasticity	601042	AD	1p21	Unknown
DYT10	Paroxysmal kinesigenic choreoathetosis	128200	AD	16p-q	Unknown
DYT11	Myoclonus dystonia	604149	AD	7q11q25	ε-Sarcoglycan
DYT12	Rapid-onset dystonia-parkinsonism	128235	AD	19q	Na+/K+ATPaseα3subunit
DYT13	Multifocal/segmental dystonia	607671	AD	1p36	Unknown
DYT15	Myoclonus dystonia	607488	AD	18p11	Unknown
DYT16	Young-onset dystonia-parkinsonism	612067	AR	2q31	PRKRA

Table2. Genetically defined forms of dystonia and dystonia-plus adapted from Klein and Ozelius (2002) and Camargos (2008). *AD* autosomal dominant, *AR* autosomal recessive, *XR* X-linked recessive.

***Pathophysiology*:** Human brain specimen of dystonia patients revealed no signs of neurodegeneration in terms of overt atrophy, neuron loss or inflammation. Yet, post mortem studies in affected DYT1 PTD patients demonstrated slightly enlarged dopaminergic cell bodies in the substantia nigra (Rostasy et al. 2003) and perinuclear ubiquitin-positive inclusions in midbrain nuclei involved in motor activity (McNaught et al. 2004). These findings could not be confirmed in other forms of primary dystonia, suggesting that they might be an epiphenomenon of the mutation rather than the general mechanism leading to a dystonic phenotype (Holton et al. 2008). In secondary dystonias, e.g. following cerebral ischemia or in spinocerebellar syndromes, regions affected by the disease can be identified neuropathologically. These indicate that symptoms in primary dystonias may result from malfunctions in the basal ganglia, cerebellum, brainstem or parietal lobe (Geyer and Bressman 2006).

MRI studies of dystonia showed enlarged basal ganglia, increased grey matter density of sensorimotor areas, and altered activity patterns in motor areas during motor tasks (Draganski et al. 2003; Defazio et al. 2007). Transcranial magnetic stimulation (TMS) studies observed an increase in motor cortex excitability potentially resulting from impaired inhibitory signals (Ikoma et al. 1996). In PET studies, D2 dopamine-receptor binding was altered in the putamen pointing to dopaminergic abnormalities in dystonia (Perlmutter et al. 1997).

Altered brain plasticity processes seem to be a major contributing factor in the pathophysiology of dystonia. Most patients suffering from task-specific dystonia like writer's cramp or musician's dystonia report an overuse of the affected body region precedent to the onset of symptoms (Quartarone et al. 2006). In these patients, somatotopic organization of motor and sensory areas is disorganized, sensorimotor inhibition is impaired and the formation of new synaptic connectivity is facilitated as revealed by transcranial brain stimulation. The fact that af-

fected DYT1 PTD patients and non-affected mutation carriers show impairments in motor learning paradigms further indicates a central role of altered plasticity processes (Ghilardi et al. 2003).

The efficacy of anticholinergic, GABAergic and dopaminergic agents is consistent with the alterations in these neurotransmitter systems. In addition, the effect of deep brain stimulation of the globus pallidus and, historically, of surgical pallidotomy also indicates the contribution of the basal ganglia in the pathophysiology. However, it cannot be concluded that these are the primary site of malfunction, since pallidal stimulation might simply override symptoms that are generated in upstream motor control systems (Breakefield et al. 2008).

DYT1 mutation: It is not known whether different types of dystonia share a common pathophysiology. Therefore, in this study we focused on DYT1 PTD which accounts for 70% of all cases of early-onset dystonia. Symptoms occur in 30-40% of all mutation carriers (reduced phenotypical penetrance) and start between 5 to 28 years of age. Triggers or risk factors for the onset are not known. Patients show no cognitive impairments and most retain the ability to walk with a more or less pronounced handicap (Kamm 2006). Mutation carriers older than 28 are virtually free from the risk of developing symptoms (Bressman 2004). However, they show, like their affected relatives, an endophenotype of altered resting metabolic activity in the basal ganglia (Eidelberg et al. 1998) and slowed motor learning (Ghilardi et al. 2003).

The DYT1 gene: The DYT1 mutation consists of an inframe deletion of a GAG triplet in the DYT1 gene (also called Tor1A) on chromosome 9q34 (Ozelius et al. 1997). DYT1 codes for the 332 amino acid protein torsinA (37.8kDa, fig. 2). The GAG deletion results in the loss of one of two adjacent C-terminal glutamic acid residues (number 302 and 303). TorsinA possesses a putative ATP-binding domain and an N-terminal signal sequence redirecting the protein to the endoplasmic reticulum (ER, Kustedjo et al. 2000). TorsinA has been attributed to the AAA+ (ATPase associated with a variety of cellular activities) protein superfamily (Ozelius et al. 1999).

DYT1 shares more than 70% homology with the gene Tor1B coding for torsinB. Two additional members of the human Tor1A family are Tor2A on chromosome 9 and Tor3A on chromosome 1, encoding torp2A (~40kDa) and torp3A (~40 or 48kDa, alternative splicing), each with about 50% homology to torsinA and with as yet undetermined functions (Ozelius et al. 1999).

The DYT1 gene including its GAG deletion site is highly conserved in species including C.elegans, drosophila and rodents which probably implies a functional significance. All members of the torsinA family show a distant relation to the eukaryotic heat shock protein 100 family. Its members act as molecular chaperones: They are upregulated during oxidative stress in the cell and are able to undo protein aggregation (Maurizi and Xia 2004).

Wildtype torsinA functions: In humans, torsinA is expressed ubiquitously in all tissues; in the CNS, expression can be detected in all neurons, but not in glial cells (Ozelius et al. 1997). TorsinA mRNA and protein levels are highest in dopaminergic neurons of the pars compacta of the substantia nigra (SNc), in the striatum, in Purkinje cells of the cerebellum and in noradrenergic cells of the locus coeruleus – similarly in humans, rats and mice (Augood et al. 1998; Augood et al. 1999; Shashidharan et al. 2000; Konakova et al. 2001; Konakova and Pulst 2001; Andersen 2003; Augood et al. 2003). On the subcellular level, torsinA is found in the lumen of the ER and enriched in presynaptic terminals (Hewett et al. 2000; Augood et al. 2003; Hewett et al. 2003). TorsinA is upregulated in response to oxidative stress, but – unlike heat shock proteins – not in response to other types of stressors (Hewett et al. 2003).

Effects of mutant torsinA: When overexpressed in a neuronal mouse cell line, mutant, but not wildtype torsinA led to perinuclear inclusions and flattened cell shapes (Hewett et al. 2000; Kustedjo et al. 2000). Perinuclear inclusions have also been found in brain tissue of DYT1 patients in the SNc and the locus coeruleus. Aggregates were positive for torsinA, ubiquitin (a

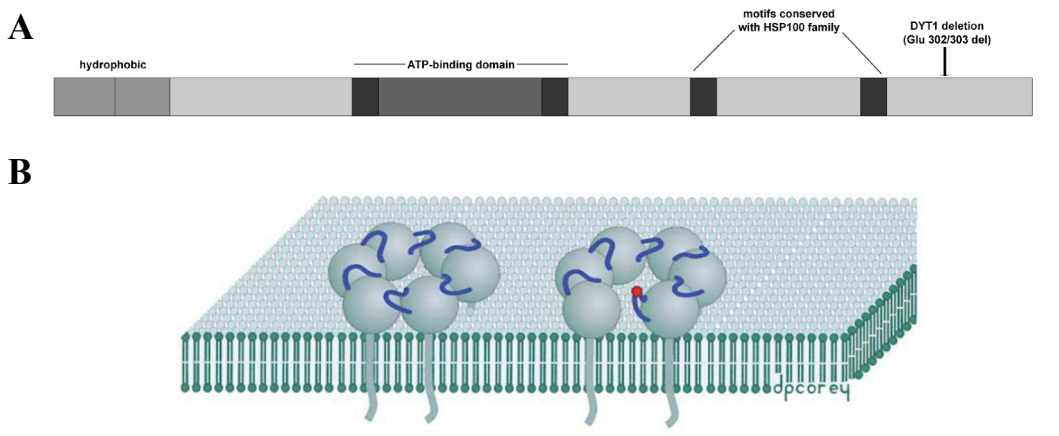


Figure 2. **The torsinA protein.** (A) Schematic representation of the torsinA protein domains with about 40 hydrophobic amino acids in the N-terminal region, the ATP-binding domain flanked by its two conserved motifs and two additional motivs conserved with the HSP 100 family. (B) TorsinA monomers predicted to form six-membered ring structures within the ER. Loss of the C-terminal glutamic acid residue (red) might interrupt protein structure or interaction with partners. Adapted from Leung et al. (2001) and Breakefield et al. (2001).

mediator of post-translational protein modification and degradation) and for the nuclear envelope marker lamin A/C consistent with the findings in cell culture. Despite of these inclusions, no signs of neurodegeneration and no signs of inflammation were present in these patients (McNaught et al. 2004).

In primary fibroblasts of DYT1 patients, torsinA was abnormally localized around the nucleus and impaired secretory processes (Hewett et al. 2006). Specific knock-down of mutant torsinA by siRNA as well as additional overexpression of wildtype torsinA restored this function to normal (Hewett et al. 2008). This indicates a dominant-negative role of the mutant protein, but up to now, implicated mechanisms and pathways are not known.

Animal models of dystonia: Surprisingly, all existing animal models that show a dystonic phenotype carry spontaneous mutations in genes that are not known from human dystonia. Nevertheless, some parallels to the human disease have been found in their analyses. The dt/dt mouse, for example, is characterized by loss of the hemidesmosomal protein dystonin resulting in an autosomal recessive phenotype with dystonic postures and neuronal degradation. Dystonin has been found to interact with the cytoskeleton and with the nuclear envelope which reminds of the functions proposed for torsinA (Raike et al. 2005; Breakefield et al. 2008).

Further insight into the pathophysiology of human hereditary dystonias has been provided by animal models of DYT1 (for which most of the work has been done), DYT5, DYT11 and DYT12. An advantage of modeling DYT1-associated dystonia is the high conservation of this protein (Raike et al. 2005). Main strategies have been knock-in, knock-out and transgenic approaches; a comprehensive summary is shown in table 3 (Zhao et al. 2008b).

Animals totally lacking the wildtype allele (i.e., $Tor1A^{-/-}$, $Tor1A^{\Delta GAG/\Delta GAG}$ and $Tor1A^{\Delta GAG/-}$) were postnatal lethal due to feeding unability. This suggested a specific failure in executing motor actions since all structures of the CNS in these mice were normally developed (Goodchild et al. 2005).

Heterozygous knock-in mice showed hyperactivity and performed worse in fine motor coordination tests. Immunohistologically, ubiquitin- and torsinA-positive aggregates were found in the pontine nuclei of males only and neurochemically, the dopamine metabolite homovanillic acid (HVA) was slightly reduced in the striatum (Dang et al. 2005). Consistently, DYT1 knock-down mice with reduced expression of the DYT1 gene showed similar behavior and neurochemistry results as heterozygous knock-in mice (Dang et al. 2006).

Partly different morphological, behavioral and neurochemical results were obtained in three transgenic murine DYT1 models that have been published so far. Human mutant (ΔGAG) torsinA driven by the neuron-specific enolase (NSE) promoter led to pathological and behavioral abnormalities in a subset of animals (Shashidharan et al. 2005). Human cytomegalovirus (CMV) promoter-driven torsinA transgenic mice showed no histological abnormalities, but minor changes of striatal dopamine and acetylcholine neurotransmission (Sharma et al. 2005; Pisani et al. 2006; Balcioglu et al. 2007; Zhao et al. 2008b).

Finally, in a third model which was used for this study, transgenic human mutant (hΔGAG, hMT) or transgenic human wildtype torsinA (hWT) was overexpressed under control of the murine prion protein promoter (Grundmann et al. 2007). The hWT group is considered as a more suitable control compared to wildtype animals. Both lines showed neuropathological, and neurochemical abnormalities as well as minor changes of spontaneous behavior (compare table 3). In both lines, motor circuit integrity as measured by diffusion tensor imaging was found to be impaired in striatum, cerebellum and motor cortex.

In summary, results from transgenic torsinA models show that mutant human torsinA causes altered levels of striatal dopamine and its metabolites and possibly also hyperactivity.

Model	Construct	Morphology	Behavior	Neurochemistry
DYT1 transgenic (Shashidharan et al. 2005)	7.1 kb fragment from the pNSE-Ex4 vector containing the neuron-specific enolase promoter, human mutant (ΔGAG) torsinA-cDNA and SV40 polyA signal	Ubiquitin- and torsinA-IR perinuclear aggregates and inclusion bodies in the peduncu-lopontine nucleus, pons and periaqueductal gray	40% of transgenic mice from each line displaying dystonic movements of limbs with self-clasping, circling behavior, and hyperactivity	↓ striatal DA in transgenic animals that exhibited an abnormal behavioral phenotype ↓ striatal DOPAC/DA ratio in all transgenic mice
DYT1 transgenic (Sharma et al. 2005; Pisani et al. 2006; Balcioglu et al. 2007; Zhao et al. 2008b)	Human wild-type (hWT) or mutant (ΔGAG) torsinA-cDNAs inserted into pcDNA3.1 under the human cytomegalovirus immediate early promoter	No torsinA-positive inclusions or increased staining around the nuclear envelope (NE)	Reduced ability to learn motor skills in an accelerating rotarod paradigm and beam-walking test	↑ striatal DOPAC/DA ratio ↓ amphetamine-induced striatal extracellular DA levels altered responses to DA2-receptor activation
DYT1 knock-in (Dang et al. 2005)	Exon 5 in the targeting vector construct carrying a GAA deletion at codon 302; PGKNeoSTOP cassette with a false translation signal, splice donor site and poly(A) tail inserted into intron 4	Ubiquitin- and torsinA-containing aggregates in pontine nuclei of male DTY1 knock-in mice	Deficient performance on the beam-walking test, open-field hyperactivity	↓ striatal HVA

Model	Construct	Morphology	Behavior	Neurochemistry
DYT1 knock-out (Goodchild et al. 2005)	Exons 2–4 of DYT1 replaced by a cassette containing Neo and IRES-tau LacZ sequences	Vesicles within the neuronal NE that appear to derive from the inner nuclear membrane	Homozygotes lethal postnatally	n/a
DYT1 knock-in (Goodchild et al. 2005)	Exon 5 in the targeting construct carries a GAG deletion. Neo cassette was inserted into intron 4 of DYT1	Homozygotes exhibiting vesicles within the neuronal NE that appear to derive from the inner nuclear membrane	Homozygotes lethal postnatally	n/a
DYT1 knock-down (Dang et al. 2006)	PGKNeoSTOP cassette with false translation signal, splice donor site and poly(A) tail inserted into intron 4 of DYT1, recombination 5′ to an Exon 5 GAA deletion	n/a	Horizontal hyperactivity, ↑ slips on a beam-walking test	↓ striatal DOPAC
DYT1 transgenic (Grundmann et al. 2007)	Human wild-type (hWT) and mutant (hΔGAG) torsinA-cDNAs inserted into pBluescript II SK- vector under 3.4 kb fragment of murine prion protein promoter and tagged C-terminally with V5-His	hWT and hΔGAG: Inclusion-like formations in brainstem nuclei, torsinA-IR localized to the NE, NE abnormalities, disrupted white matter connectivity	hWT mice: hypoactivity, short stride length, prolonged traversal times on beam-walking hΔGAG mice: hyperactive, defects on rotarod testing	hWT mice: ↓ striatal DA, serotonine and 5-HIAA; ↓ brainstem HVA. hΔGAG mice: ↑ brainstem DOPAC, serotonine and 5-HIAA

Table3. Summary of murine DYT1 models that have been described to date, adapted from Zhao et al. (2008b) and Breakefield et al. (2008). *DA* dopamine, *DOPAC* Dihydroxyphenylacetic acid, *5-HIAA* 5-Hydroxyindoleacetic acid, *HVA* Homovanillic acid, *IR* immunoreactive, *NE* Nuclear envelope.

2. Adult neurogenesis

Background: Adult neurogenesis is the generation of new neurons in the adult brain. Until the 1990s, most neuroscientists' view was that all neurons were produced during embryonic and postnatal development and that no new neurons could be generated afterwards: "In the adult nerve centers, the nerve paths are something fixed and immutable: everything may die, nothing may regenerate" (Ramón y Cajal 1928). However, today it is accepted that ongoing neuronal production is found in the adult mammalian brain throughout life within two neurogenic areas. As mature neurons have not been shown to undergo cell division, the ongoing generation of mature neurons requires the presence of neural stem cells. These are defined by their ability of unlimited self-renewal by symmetric or asymmetric division. Self-renewal of neural progenitor cells is limited; they are able to rapidly proliferate, but will finally all differentiate into neurons and glial cells. Neural stem and progenitor cells are summarized by the term precursor cells since they often cannot be distinguished [reviewed by Ming and Song (2005) and Zhao et al. (2008a)].

Methods to detect newly generated neurons: In the 1960s, the first indications for the adult production of new neurons were found by [H³]-thymidine incorporation into replicating cells (Friedkin et al. 1956; Messier et al. 1958; Altman and Das 1965a). The thymidine-analog bromodeoxyuridine (BrdU) can be detected by immunohistochemistry and thus allows absolute quantification and proof of colocalization by multiple stainings (Kaplan and Hinds 1977; Gratzner 1982; Cameron et al. 1993; Kuhn et al. 1996). Before its use in neurogenesis research, it had already been established as a marker for cell proliferation *in vivo* and *in vitro* (Dolbeare 1995) since it does not influence transcription and viability of proliferating cells (Dunn et al. 1954; Zamenhof and Gribiff 1954). The sensitivity of the method depends on the applied dose of BrdU: too low doses miss dividing cells or will be diluted in subsequently dividing cells whereas too high doses overload cells and induce apoptosis. Therefore, injection paradigms usually require daily applications for 1-7 days depending on the gap between injections and tissue fixation (Cameron and McKay 2001). As drawbacks, analyses cannot be conducted in alive animals since BrdU detection needs tissue fixation and confocal microscopy is required for a proper attribution of a BrdU-positive nucleus to the neuronal-maker-positive cytoplasm (Rakic 2002). Other approaches for the detection of newborn cells include labeling by retroviruses and labeling of cells by genetic manipulations (Lewis and Emerman 1994; Tashiro et al. 2006). Neuroblasts can also be identified due to the expression of specific markers, e.g. doublecortin [DCX, (Couillard-Despres et al. 2005)].

Sites of ongoing neurogenesis in the adult mammalian brain: Neurogenesis in the intact adult mammalian brain is restricted to two neurogenic regions. Adult olfactory neurogenesis occurs in the subventricular zone (SVZ) below the ependyma of the lateral wall of the lateral ventricles where neural stem and progenitor cells reside (Fig. 2 A, B). From there, newly generated cells migrate within the rostral migratory stream (RMS) into the olfactory bulb where they terminally differentiate and integrate into the local network (Altman and Das 1965b; Corotto et al. 1993; Curtis et al. 2007). Stem and progenitor cells of adult hippocampal neurogenesis reside in the subgranular zone (SGZ) medial of the granule cell layer of the dentate gyrus (GCL; Fig. 2 A, C). Following short-distance tangential migration within the SGZ, new cells move radially into the GCL where they become new granule cells. They receive inputs on their dendrites from association cortex and entorhinal cortex areas via the perforant pathway and relay axonal output into CA3 pyramidal cell layer via the mossy fiber pathway (Altman and Das 1965a; Eriksson et al. 1998; van Praag et al. 2002).

Similar differentiation stages are present in the two neurogenic regions. In both systems, the slowly cycling putative stem cells ("B-cells" in SVZ, "type1 cells" in DG) are GFAP-, Hes5- and nestin-positive (Lendahl et al. 1990; Doetsch et al. 1999a; Doetsch et al. 1999b; Lugert et al. 2010). The derived transiently amplifying progenitor cells ("C-cells" in SVZ/RMS, "type2 cells" in DG) are GFAP-negative and downregulate nestin. Proliferating cell nuclear antigen (PCNA) is a marker for neural progenitor proliferation (Hall et al. 1990). With their specification to the neuronal lineage and onset of migration, precursor cells upregulate DCX and the polysialylated form of the neural cell adhesion molecule [PSA-NCAM; "A-cells in SVZ/RMS, "type3 cells" in DG (Tomasiewicz et al. 1993; Meyer et al. 2002)]. Doublecortin is a microtubule-associated protein found primarily in the leading processes and growth cones of neurons (Bai et al. 2003; LoTurco 2004; Schaar et al. 2004). As soon as the cells have reached their target, have become postmitotic and start extending axons and dendrites, DCX and PSA-NCAM are downregulated and mature neuronal markers like neuron-specific enolase (NSE) and neuronal nuclei (NeuN) are upregulated (Mullen et al. 1992).

***Modulation*:** A variety of endogenous and exogenous factors are regulators of adult neurogenesis. The baseline rate of new hippocampal neurons during adulthood is significantly affected by genetic background of the animal with huge differences between wild-type and inbred laboratory strains (Kempermann and Gage 2002). Besides, aging is the strongest negative regulator of adult neurogenesis in both SVZ and SGZ causing an rapid decline of the number of new surviving neurons with age (Kuhn et al. 1996; Enwere et al. 2004; Kempermann 2011). Finally, environmental stimuli also modulate adult neurogenesis, e.g. stress (Mirescu and Gould 2006), environmental enrichment and physical activity (Kempermann et al. 1997; van Praag et al. 1999). As seen in table 4, a variety of neuroactive compounds has also been shown to modulate proliferation, differentiation and survival of new neurons, with yet unclear physiological relevances (Ming and Song 2005).

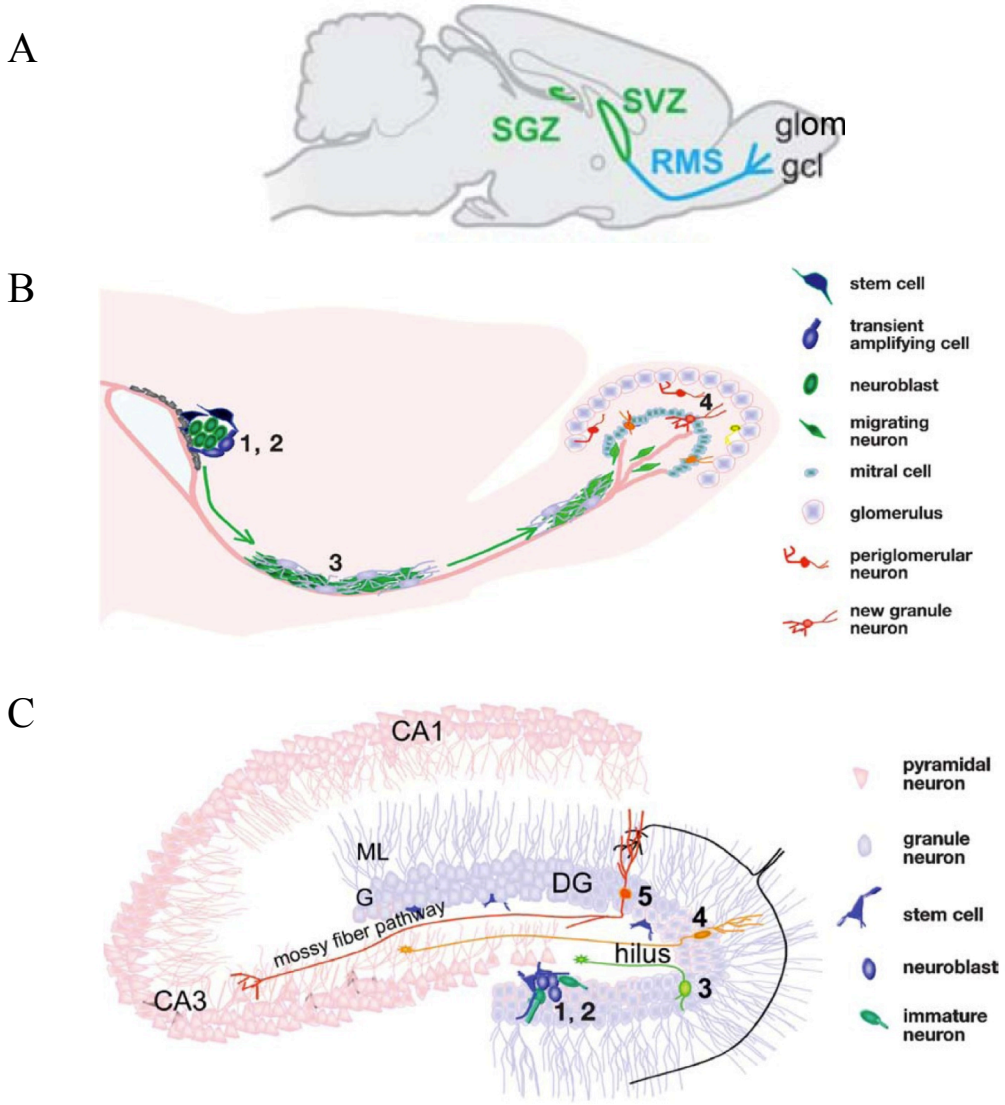


Figure 3. **Schematic drawing of the two neurogenic zones of the adult mammalian CNS.** (A) Neural stem and progenitor cells reside within the SGZ of the DG and within the SVZ of the lateral ventricles. (B) In the SVZ, the neural stem and progenitor cells proliferate (1) and differentiate (2) towards a glial or neuronal (neuroblast) fate. From there, the immature neurons migrate along the astrocyte-ensheated RMS into the OB (3) where they radially move into the GCL or the GLOM and integrate into synaptic circuits as new granule or periglomerular neurons, respectively (4). (C) In the hippocampus, stem cells proliferate within the SGZ (1). Deriving neuroblasts (2) migrate radially into the DG (3). There, the immature neurons extend their axon along the mossy fiber pathway toward the CA3 pyramidal layer and their dendrites into the molecular layer of the DG (4). After complete synaptic integration, the new neurons become mature granule cells (5). Figures adapted from Ming and Song (Ming and Song 2005).

Regulatory factors	Impact	Reference
Hormones		
Corticosterone	SGZ proliferation ↓	(Cameron and Gould 1994)
Estrogen	SGZ proliferation ↑	(Tanapat et al. 1999)
Neurotransmitters		
Dopamine	SVZ + SGZ proliferation ↑	(Baker et al. 2004; Hoglinger et al. 2004; Van Kampen et al. 2004; Winner et al. 2006)
Serotonin	SVZ + SGZ proliferation ↑	(Banasr et al. 2004)
Acetylcholine	SVZ + SGZ survival ↑	(Cooper-Kuhn et al. 2004)
Glutamate	SGZ proliferation ↓	(Nacher and McEwen 2006)
Growth factors		
FGF-2 and EGF	SVZ proliferation ↑	(Kuhn et al. 1997)
VEGF	SVZ + SGZ survival ↑	(Schanzer et al. 2004)
IGF-1	SGZ survival ↑	(Aberg et al. 2000)

Table4. General influence of different neuroactive compounds on adult neurogenesis: ↓ = decrease, ↑ = increase.

Putative functions of adult neurogenesis: Hypothetically, the integration of new neurons offers a profound form of structural plasticity, making adult neurogenesis a possible contributor to the formation of memories. In the hippocampus and the SVZ/OB, behavioral or pharmacological modulation of neurogenesis is accompagnied by changes in the performance in memory tasks and by changes in long-term potentiation (van Praag et al. 1999). Accordingly, newly generated mature neurons exhibit facilitated induction and larger amplitudes of long-term potentiation (Schmidt-Hieber et al. 2004). Computational and behavioral analyses of adult hipppocampal neurogenesis suggest a role in preventing different memories from interfering with each other (Wiskott et al. 2006) and in the separation of memories that are related in time or space (Aimone et al. 2009; Clelland et al. 2009; Deng et al. 2010; Aimone and Gage 2011; Sahay et al. 2011). Adult olfactory neurogenesis seems to be independent from its hippocampal counterpart, but has also been linked to the formation of new – olfactory – memories (Rochefort et al. 2002; Enwere et al. 2004).

Neurodegenerative diseases affecting adult neurogenesis: As indicated by the mass of regulatory influences on adult neurogenesis, profound changes have been detected in some disorders of the CNS including neurodegenerative diseases (table 5, Winner et al. (2011)).

Model	Impact	Reference
Acute neurological diseases		
Cerebral ischemia	SVZ + SGZ proliferation ↑ migration toward the lesion	(Arvidsson et al. 2002)
Temporal lobe epilepsy	SGZ proliferation + survival ↑ neuroblast dispersion in hippocampus	(Parent et al. 1997)
Inflammation	SGZ proliferation + survival ↓	(Monje et al. 2003)
Huntington's disease		
Human patients	SVZ proliferation ↑	(Curtis et al. 2003)
Lesion animal model	SVZ proliferation ↑	(Tattersfield et al. 2004)
Genetic animal models	SVZ + SGZ survival ↓	(Gil et al. 2004; Lazic et al. 2004; Gil et al. 2005; Kohl et al. 2010)
Alzheimer's disease		
Human patients	SGZ proliferation ↑	(Jin et al. 2004b)
Genetic animal models	SGZ/SVZ proliferation/survival ↓↑	(Haughey et al. 2002a; Haughey et al. 2002b; Jin et al. 2004a; Boekhoorn et al. 2006)
Parkinson's disease		
Human patients	SVZ + SGZ proliferation ↓	(Hoglinger et al. 2004)
Lesion animal models	SVZ proliferation ↓	(Baker et al. 2004; Winner et al. 2006)
Genetic animal models	SGZ/SVZ proliferation/survival ↓/↔	(Winner et al. 2004; Nuber et al. 2008; Winner et al. 2008b; Marxreiter et al. 2009)

Table 5. Influence of different brain pathologies and neurodegenerative diseases on adult neurogenesis in animal models and humans: ↓ = decrease, ↑ = increase.

3. Potential effects of transgenic torsinA on adult neurogenesis

The aim of this work was to investigate potential changes in the population of adult neural precursors concerning their proliferation and survival in the transgenic hWT and hMT mouse models (Grundmann et al. 2007).

Hypotheses: For further insight into the role of adult neurogenesis in DYT1, we addressed the following hypotheses and respective aims:

I. In hMT and hWT mice, transgenic torsinA is overexpressed in the neurogenic regions.
Aim: Immunohistochemical detection of transgenic and endogenous torsinA in the SVZ, OB and DG.

II. Transgenic wildtype or mutant torsinA is expressed within neural progenitor cells during migration and differentiation.
Aim: Colocalization analysis of transgenic torsinA and markers of different stages of neuroblast differentiation.

III. DYT1-related striatal pathology leads to ectopic neuroblast migration.
Aim: Qualitative analysis of the distribution of newly generated neurons and quantification of striatal neuroblasts.

IV. Transgenic wildtype or mutant torsinA impairs adult neurogenesis.
Aim: Quantification of proliferation, survival and differentiation of newly generated neurons in the hippocampus and the SVZ/OB.

Transgenic expression of torsinA: In the initial characterization of these mouse models, torsinA expression was highest in pons, brainstem, cerebellum and the OB (Grundmann et al. 2007). Weaker immunostaining was observed in the striatum and the hippocampus. Therefore, it is important to first confirm the expression of torsinA in the neurogenic areas of these mouse models (aim I).

DYT1 has cell-autonomous effects on adult neurogenesis: TorsinA expression in mice and rats is highest from embryonic day 15 to postnatal day 14 and shows a site-specific correlation with the peaks in neurogenesis, neuronal migration and initial synaptic formation in striatum and hippocampus (Xiao et al. 2004; Vasudevan et al. 2006). As expression levels of torsinA rapidly decrease thereafter until postnatal day 30, it is thought to exert functions in neural development. Accordingly, torsinA was shown to regulate neuronal migration during embryonic development (Bhide et al. 2008). Therefore, it is important to examine if in the mouse models, transgenic torsinA is overexpressed also within adult neural progenitor cells (aim II)

Striatal neuroblast migration: Under different pathological conditions, e.g. models of Huntington's disease and ischemia and following the application of growth factors, high DCX-expression has been shown in the striatum in association with newly generated cells. These cells were migrating from the SVZ into the diseased striatum with or without ending in neuronal maturity (Arvidsson et al. 2002; Winner et al. 2008a; Kohl et al. 2010). Striatal dysfunction is a hallmark of DYT1-dystonia patients and has been demonstrated by MRI and TMS (Perlmutter et al. 1997; Draganski et al. 2003; Perlmutter and Mink 2004; Defazio et al. 2007). In hWT and hMT mice, striatal pathology includes altered neurotransmitter levels and

disrupted white matter connectivity (Grundmann et al. 2007). Consequently, in hWT/hMT mice, neuroblasts might also be redirected into the diseased striatum (aim III).

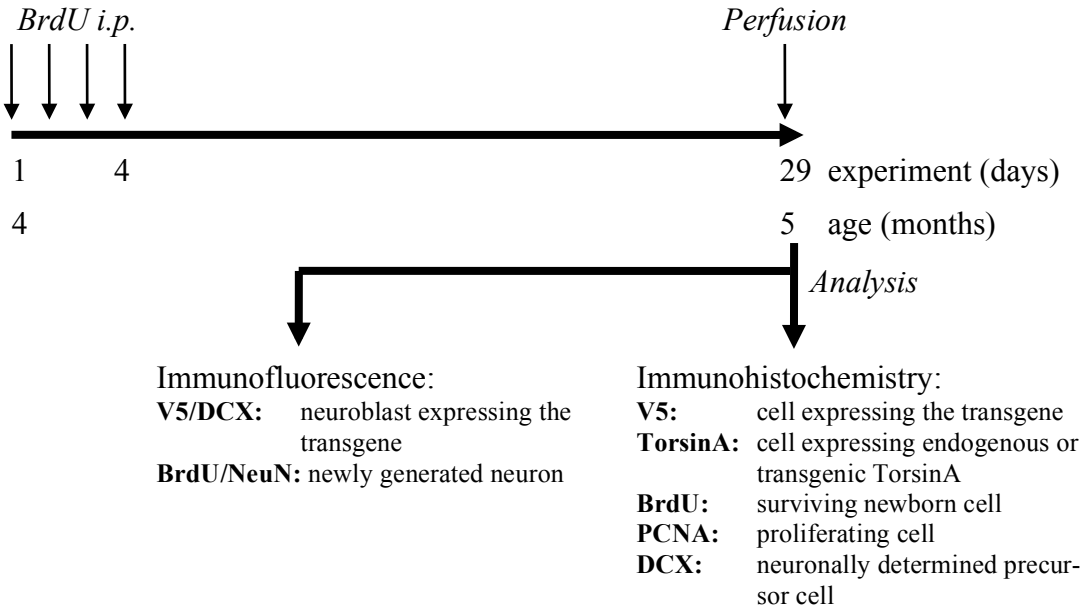
Intraneuronal torsinA inclusions affecting neural progenitors: Intraneuronal aggregates were observed in previously characterized transgenic animal models overexpressing human α-synuclein. In these models, survival of newly generated neurons is decreased in the OB and in the SGZ, correlating with increased cell death in these regions (Winner et al. 2004; Nuber et al. 2008; Marxreiter et al. 2009). TorsinA-positive inclusions are a histopathological feature in DYT1 patients and in the hWT/hMT mouse models (McNaught et al. 2004; Grundmann et al. 2007). Therefore, torsinA might lead to reduced survival of newborn neurons due to the formation of intraneuronal inclusions (aim IV).

Cell non-autonomous torsinA leads to reduced neurogenesis: Aside from an endogenous influence on adult neural precursor cells, the DYT1 mutation might also influence adult neurogenesis by a cell non-autonomous mechanism. TorsinA is highly expressed in the OB of hWT and hMT mice; thus, the "diseased" environment could lead to impaired integration and survival of newborn neurons (aim IV).

DYT1 alters neurogenesis due to altered dopamine or hyperactivity: Imbalances of the striatal dopaminergic system have been found both in PTD patients and in DYT1 animal models. Lack of striatal dopamine is an established downregulator of adult SVZ neurogenesis both in animal models and PD patients whereas excess striatal dopamine seems to have almost no upregulatory effect (Borta and Hoglinger 2007). Therefore, DYT1-induced changes in striatal dopamine might affect the rate of SVZ neurogenesis as a cell-non-autonomous effect. In the hWT mouse model, striatal dopamine levels are decreased which is therefore expected to exert a negative impact on SVZ neurogenesis (aim IV).
In addition to neurochemical changes within the brain, the hyperactive phenotype of DYT1 transgenic mice as a form of physical activity could cause a secondary increase of adult hippocampal neurogenesis. As the survival-promoting effect of physical activity is specific for the hippocampus, it would have no influence on SVZ/OB neurogenesis (aim IV).

C. Materials and Methods

The experimental part of this work was conducted in the laboratories of the department of neurology of the university of Regensburg according to the following paradigm:



1. Animals

A total of 21 C57BL/6N mice were provided by the Department of Medical Genetics at the University of Tübingen, Germany which has previously characterized them (Grundmann et al. 2007). Six of them were control animals, eight were transgenic for human mutant torsinA (hMT group) and seven were heterozygously transgenic for human wildtype torsinA (hWT group).

In brief, transgenic animals had been generated by means of a vector consisting of the murine prp promoter, the human DYT1 sequence (wildtype or mutant) to be expressed under the control of this promoter and the V5-His tag as a marker. The construct had been injected into the pronucleus of a fertilized egg produced by a superovulated female mouse. It randomly integrates into the DNA by non-homologous recombination. Microinjected oocytes are transferred into pseudo-pregnant females mated with vasectomised males. Less than 20% of the offspring are founder mice containing the foreign DNA in all their tissues and germ lines (founder mice). Breeding had been continued for a few generations to ensure that the

transgene is repetitively propagated within the germ line and to achieve genetic uniformity (Lodish 2008).

Genotypes were determined by ear biopsy PCR. Genomic DNA was isolated from the tissue. Human wildtype and human mutant torsinA-cDNA were positive controls and biopsy material of nontransgenic animals were negative controls. DNA was isolated using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Hamburg, Germany). With the primer 3696_PrP_F (TTTGGAATATGTTTGCGCTG) combined with 2026_torA_seq1_R (TGTTCAGACCACCCTCGTAA) or 2028_torA_seq2_R (GCTTGATGTCTTCCCTCTGC), a 600bp segment or a 830 bp within the human torsinA coding sequence was amplified according to the following conditions: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, 35 cycles (Grundmann et al. 2007).

In each group, genders were distributed equally (hMT: 4 ♂, 4 ♀; hWT: 4 ♂, 3 ♀; control: 3 ♂, 3 ♀). One male animal of the control group died during the experimental phase for unknown reasons, resulting in a control group size of 5.

The inserted constructs consisted of the murine prion protein gene promoter including exon 1, intron 1 and part of exon 2 which are necessary to promote a high rate of transcription (Baybutt and Manson 1997). Constructs contained the genes encoding either human wildtype torsinA (hWT group) or human mutant torsinA (hMT group) followed by a V5-His tag (Fig. 4). The V5-His tag is a marker protein for visualization of the transgene. The V5-epitope is a 14 amino acid peptide and the attached His tag is a 6 amino acid polyhistidine sequence.

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local governmental commission for animal health. Animals were kept in normal light dark cycles of 12h in standard laboratory cages and had free access to food and water.

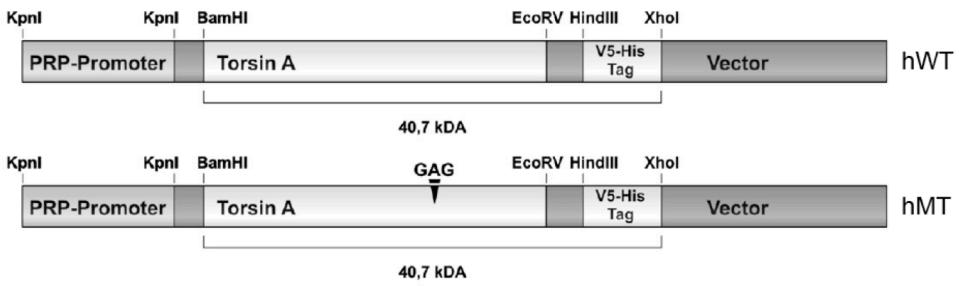


Figure 4. **The two constructs used for overexpressing human wildtype torsinA (hWT) or human mutant torsinA (hMT)** under control of the murine prion protein promoter (prp-promoter). Both constructs are identical except for the deletion of the GAG-triplet in hMT. Reprinted from Grundmann et al. (2007).

2. BrdU labeling and tissue processing

BrdU labeling: Powdery 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, Steinheim, Germany) was completely dissolved at 10mg/mL in sterile saline (0,9% w/v NaCl) under stirring and heating. From days 1 to 4, all animals received daily intraperitoneal injections of BrdU (50mg BrdU per kg body weight) to label dividing cells.

Brain processing: 28 days after the first BrdU injection, all animals were sacrificed. 0.4mL of a mixture of anaesthetics consisting of ketamine [Ketanest®] (20.38mg/mL), xylazine [Rompun®] (5.38mg/mL) and acepromacin [Vetranquil®] (0.29mg/mL) dissolved in sterile saline (0.9% w/v NaCl) was applied intraperitoneally. When animals were completely anesthetized and showed no more pain reflexes, median sternotomy was performed and the heart was uncovered. The left ventricle was incised at the apex and an atraumatic perfusion needle was inserted into the aorta. NaCl (0.9% w/v) was applied via the perfusion needle for 10 minutes followed by 15 minutes of 4% paraformaldehyde to fix tissue. Then, skull and superior vertebral canal were opened and brains including hindbrain and medulla were carefully dissected. After separating brains and spinal cords, they were stored overnight in 4% paraformaldehyde for complete penetration of the tissue. Finally, tissue was stored in a solution of 30% sucrose in 0.1M PO_4-buffer at 4°C.

Microtomy: For the creation of tissue sections with a thickness of 25µm, brains were cut into two hemispheres – left hemispheres of 2 animals per group were used for coronal sections ranging from forebrain (excluding the OB) to midbrain while the remaining left hemispheres and all of the right hemispheres were utilized for sagittal sections ranging from the olfactory bulb to cerebellum and hindbrain. Tissue sections were obtained on a sliding microtome (SM2000R, Leica, Wetzlar, Germany) set to a thickness of 25µm and cooled by dry ice. Sections were stored in cryoprotectant solution (25% ethylene glycol, 25% glycerol in 0.1M phosphate buffer, pH 7.4) at 4°C.

3. Immunohistochemistry

Principles of immunohistology: For immunohistological detections, a primary antibody is used first to specifically bind to the desired antigen followed by the application of a secondary antibody that is chemically coupled with an enzyme or a flourochrome and that is raised in a different species. In enzymatic immunohistochemistry, an enzyme transforms the added chromogen into an insoluble, stable product and thus provides a color signal at the location of the antigen. The most widely used enzymes are horseradish peroxidase (HRP) with the chro-

mogen 3,3′-diaminobenzidine (DAB) generating a brown precipitate and alkaline phosphatase (AP). For immunoflourescence stainings, secondary antibodies are directly coupled to a flourochrome that emits light at a certain wavelength after excitation. The most common flourochromes are fluorescein isothiocyanate (FITC) which is excited at 490nm and emits at 550nm (green) and tetramethyl-rhodamineisothiocyanate (TRITC) which is excited at 520-554nm and emits at 582nm (red). Diamino-phenylindole (DAPI) or propidiumiodide (PI) are used as fluorescent nuclear counterstains (Lang 2006). Signals can be increased, for example by a biotin-streptavidin kit. A biotin-coupled secondary antibody has to be used and in an intermediate step, a mixture of enzyme- or fluorochrome-coupled biotin and excess streptavidin binds to biotin on the secondary antibody. As avidin has four binding sites for biotin, a "lattice" of biotin and streptavidin is formed and a multitude of enzymes or fluorochromes will be bound to the secondary antibody resulting in a higher signal (Hsu et al. 1981).

Immunoperoxidase stainings: In this work, the following primary IgG-antibodies and final dilutions were used for enzymatic immunostainings: monoclonal antibody against BrdU derived from rat in a dilution of 1:500 (rat α-BrdU monoclonal, Oxford Biotechnology, Oxford, UK), mouse α-NeuN (1:500), goat α-DCX polyclonal (1:250), mouse α-PCNA monoclonal (1:500, both Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse α-V5 (1:1000, Invitrogen, Carlsbad, CA, USA), mouse α-torsinA (1:50, D-M2A8, Cell Signaling Technology Inc., Danvers, MA, USA). Goat α-DCX polyclonal was directed against the C-terminal part of the human doublecortin protein, but also detects mouse and rat doublecortin because of the high homology between the species. Mouse α-torsinA antibody had been generated by immunization with an N-terminal fusion construct of amino acids 51-324 of torsinA with the maltose binding protein (Hewett et al. 2000; Hewett et al. 2003; Hewett et al. 2004). The following secondary antibodies were applied: donkey α-rt-IgG biotinylated, donkey α-goat-IgG biotinylated and donkey α-mouse-IgG biotinylated (all diluted 1:500, Jackson Immuno Research, West Grove, PA, USA).

Free-floating sections were transferred from the cryoprotectant solution into tris-buffered saline (TBS: 0.15M NaCl, 0.1M Tris–HCl, pH 7.4 in dH_2O) and repeatedly washed in TBS. Next, they were treated with 0.6% H_2O_2 (Merck KGaA, Darmstadt, Germany) in TBS for 30min at room temperature to abolish any endogenous peroxidase activity within the tissue that could transform the chromogen resulting in a false-positive signal. In a blocking step after another washing step in TBS, sections were incubated in 3% normal donkey serum (PAN Biotech GmbH, Aidenbach, Germany) and 0.1% Triton-X-100 (Sigma, Steinheim, Germany) in TBS for 30min at room temperature. By this means, binding sites of mouse tissue that react

unspecifically with donkey-derived immunoglobulins are occupied by unlabeled antibodies. Tissue was transferred into a solution of the primary antibody (rat α-BrdU, goat α-DCX, mouse α-PCNA, mouse α-V5 or mouse α-TorsinA) diluted in blocking buffer. After an incubation time of 12h at 4°C and a washing step in TBS, the secondary antibody diluted in blocking buffer was applied for 1h at room temperature. Following another washing step in TBS, avidin-biotin-peroxidase complex (1:100 in TBS, Vectastain Elite PK-6100, Vector Laboratories, Burlingame, CA, USA) was applied for 1h at room temperature. Finally, peroxidase detection was performed for 7min with 25mg/mL 3,3′-diaminobenzidine in 0.01% (v/v) H_2O_2 and 0.04% (w/v) $NiCl_2$ (DAB peroxidase substrate kit SK-4100, Vector Laboratories). The reaction was stopped by rinsing in tap water and free-floating sections were stored in TBS. Sections were mounted onto object glasses (Superfrost Slides, Menzel, Braunschweig, Germany). To dehydrate the tissue, object glasses were repetitively dipped into the organic xylene-substitue NeoMount (Merck KGaA). Finally, they were coverslipped with the cover medium NeoMount and a coverglass (24x60mm, Menzel).

DNA-denaturation: As the two markers BrdU and PCNA are antigens located within the cell nucleus, additional steps had to be taken before the blocking step to break down the nuclear membrane and to make the DNA accessible by denaturation (Brown et al. 2003a): sections were incubated for 2h in 50% formamide (Merck KGaA) and 2x SSC (0.3M NaCl, 0.03M sodium citrate) at 65°C, followed by rinsing for 5min in 2x SSC at room temperature. Then, sections were incubated for 30min in 2M HCl at 37°C to denaturate DNA, and were rinsed for 10min in 0.1M boric acid, pH 8.5, at room temperature. After several rinses in TBS, staining was continued with the blocking step.

Antigen enhancements: For the mouse α-torsinA antibody, an antigen retrieval method had to be conducted to allow enhanced penetration (Shi et al. 1991; Gown et al. 1993). Before the hydrogen peroxide step, sections were mounted on gelatine coated object glasses (Engelbrecht, Edemünde, Germany) and dried. An ascending alcohol row (20%, 50%, 70%, 95%, 100%; 2min each) was performed for dehydration, slides were dried again and transferred into the antigen retrieval buffer (Citra Solution, BioGenex, San Ramon, CA, USA) diluted 1:10 in 0.01M citrate buffer pH 6.0. In a microwave oven, the container was heated at 900W for 3min until it came to a rapid boil. After cooling down for 10min, another heating and a final cool down for 30min, a descending alcohol row was performed. Standard staining protocol was conducted with solutions pipetted directly onto the slides with sections surrounded by a oily line (Dako Pen, Dako, Glostrup, Denmark).

Prolongated incubation times: For the mouse α-V5 antibody, sufficient staining was achieved by extending incubation time of the primary antibody to 48h.

Immunofluorescensce staining: For immunofluorescence stainings, the same protocol as in the immunoperoxidase stainings was conducted except for the hydrogen peroxide treatment. Two different antibodies were applied at the same time diluted in blocking buffer. After 48h of incubation and three washing steps in TBS, slides were incubated in the two respective secondary antibodies for another 12h: donkey α-mouse-IgG-488, donkey α-rat-IgG-568 or donkey α-goat-IgG-568 (all 1:1000, Molecular Probes, Eugene, OR, USA). Following steps were performed in the dark to avoid photobleaching. On the third day, sections were washed in TBS followed by 10min treatment with 0.5µM ToPro-3 (Molecular Probes) diluted in TBS to label nuclei. ToPro-3 is an intercalating agent that emits light at 661nm. Sections were mounted on object glasses (Superfrost Slides, Menzel) and coverslipped in Prolong Gold Antifade Reagent (Invitrogen, Eugene, OR, USA).

4. Microscopy

To evaluate the samples, different types of microscopes were used: Quantitative cell counting and volume analyses were performed at a Leica light microscope (Leica Microsystems GmbH) connected to a semiautomatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, VT, USA) controlling the x- and y-stage position.

For fluorescence microscopy, a confocal scanning laser microscope (Leica TCS-NT, Bensheim, Germany) was used. Laser light passing two confocally set pinholes excites one single focal point and therefore allows specific visualization of one z-layer. Probes are automatically line scanned to receive a complete picture of one layer in the z-plane. Sources of false-positive signals in fluorescence microscopy include autofluorescence (from endogenous components of the cells) and crosstalk (from simultaneous recording). Autofluorescence was excluded by the control stainings showing no positive signal. Crosstalk was observed as seen in Fig. 5; therefore, all confocal images were recorded sequentially (Pawley 2005).

False-positive double labeling due to an overlay of signals from different cells was excluded by moving through the z-axis. Pictures were aquired in 1024x1024 pixels format with 8 averaged shots on a 25x PL FLUOTAR oil objective (0.75 numerical aperture) or a 40x PL APO oil objective (1.25 numeric aperture). Pinhole setting was 105µm and corresponded to a focal plane of 2mm or less.

A light microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with 5x, 10x, 20x, 40x and 100x objectives and a color video camera (Diagnostic Instruments, Sterling

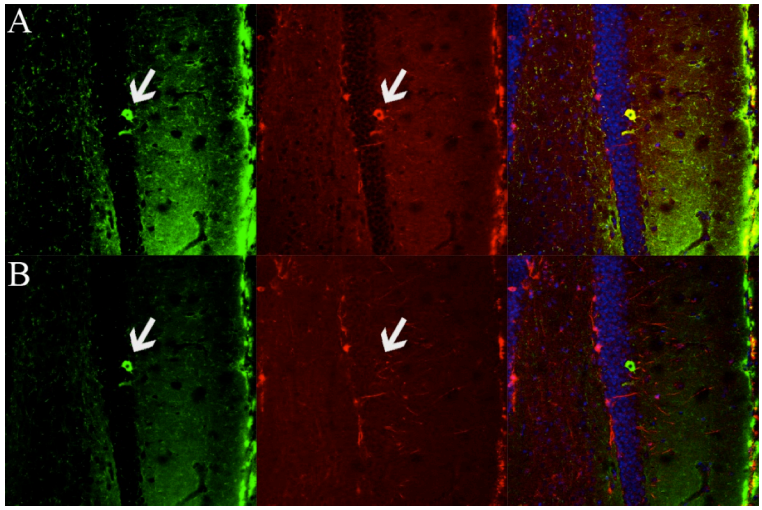


Figure 5. **Confocal laser scanning microscopy** images with (A) simultaneous scanning in all three channels produces substantial crosstalk resulting in false-positive colocalization signal whereas sequential scanning (B) allows reliable separation of the signals.

Heights, MI, USA) was used for qualitative examinations of the DAB stainings via the connected software (SPOT Advanced 3.5.4, Diagnostic Instruments, assembled by Visitron Systems GmbH, Puchheim, Germany). Illumation settings were adjusted to produce an image as close to the optical impression as possible. Gamma-correction was set to 0.8, noise filter to 10% and chip defect correction to on.

Anatomical regions were defined according to a reference atlas (Paxinos and Franklin 2004). All images were captured with each channel representing one fluorophore signal and were post-processed in Adobe Photoshop CS2 V9.0 (Adobe, San Jose, CA, USA). For pictures of the DAB stainings, the red color channel was converted into a black and white picture. If contrasts were too low, color levels were optimized without alienating the original image.

5. Stereological Counting

BrdU-positive cells were counted within the DG (including the SGZ) and the GCL and the GLOM. The researcher was blind to the genotype in order to avoid bias. For quantification of BrdU-labeled cells, design-based stereology was used which is a statistical method for estimating total cell numbers within a three-dimensional structure. It is based on the evaluation of tissue sections of equal distance covering this structure. As every sixth section (with equal distance of 150µm) was evaluated, artifacts due to cutting-derived shrinkage of tissue are compensated. Cells intersecting the uppermost focal plane (exclusion plane) were not counted. Total numbers of labeled cells as well as total volumes were obtained by multiplication with six (Schmitz and Hof 2005). Additionally, cell per volume (in μm^3) densities were calcu-

lated by dividing total cell number by total volume. As both OB and hippocampus are located symmetrically within both hemispheres, total numbers given in this work must be doubled to obtain total numbers for both sides.

The semi-automatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, VT, USA) consisted of a light microscope with a black and white video camera and an automated stage control. Brain regions can be marked and measured on the screen. Also, the field of vision and the objective can be shifted without losing selections. First, reference volumes were determined by tracing the areas under the 20x objective. Total cell numbers in the DG and the GLOM were counted manually. The vast cell numbers in the GCL of the OB of one section were estimated with the optical fractionator method (West et al. 1991) using a virtual counting frame (Williams and Rakic 1988): the manually outlined GCL regions were overlaid by a grid and only parts, the counting frames, were quantified. Its left and bottom boundary were excluded (Fig. 6). Obtained counting numbers were multiplied with the reciprocal value of the sampling probability, i.e. the proportion of the counting frame area to one grid area. Counting frame size was 30x30µm² spaced in a grid size of 300x300µm² resulting in a ratio of one counted cell per 100 estimated cells of the GCL. PCNA-positive cells were manually counted in the SGZ / DG only as no differences were expected for the SVZ due to the equal numbers of BrdU-positive cells in all groups (see results).

6. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) which reflects accuracy of the mean. As three different unmatched groups were to be compared, statistical analysis for the BrdU- and PCNA-positive cell numbers was performed using the one-way analysis of variance (ANOVA) which detects differences in the means of the groups using Prism (GraphPad Software Inc., San Diego, CA, USA). As a post-test, Tukey test was conducted to compare all pairs of groups. Significance level was set at $p < 0.05$.

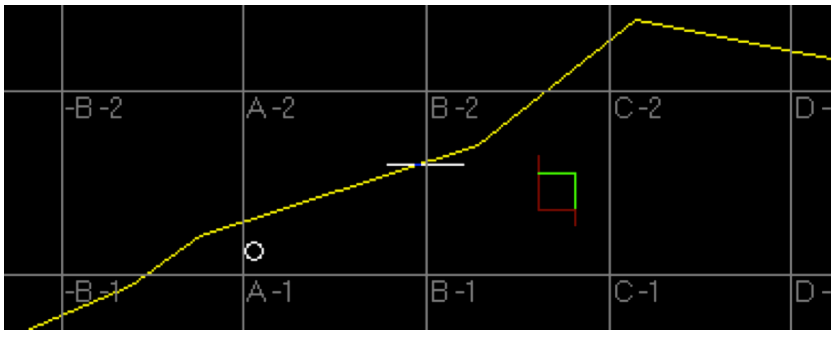


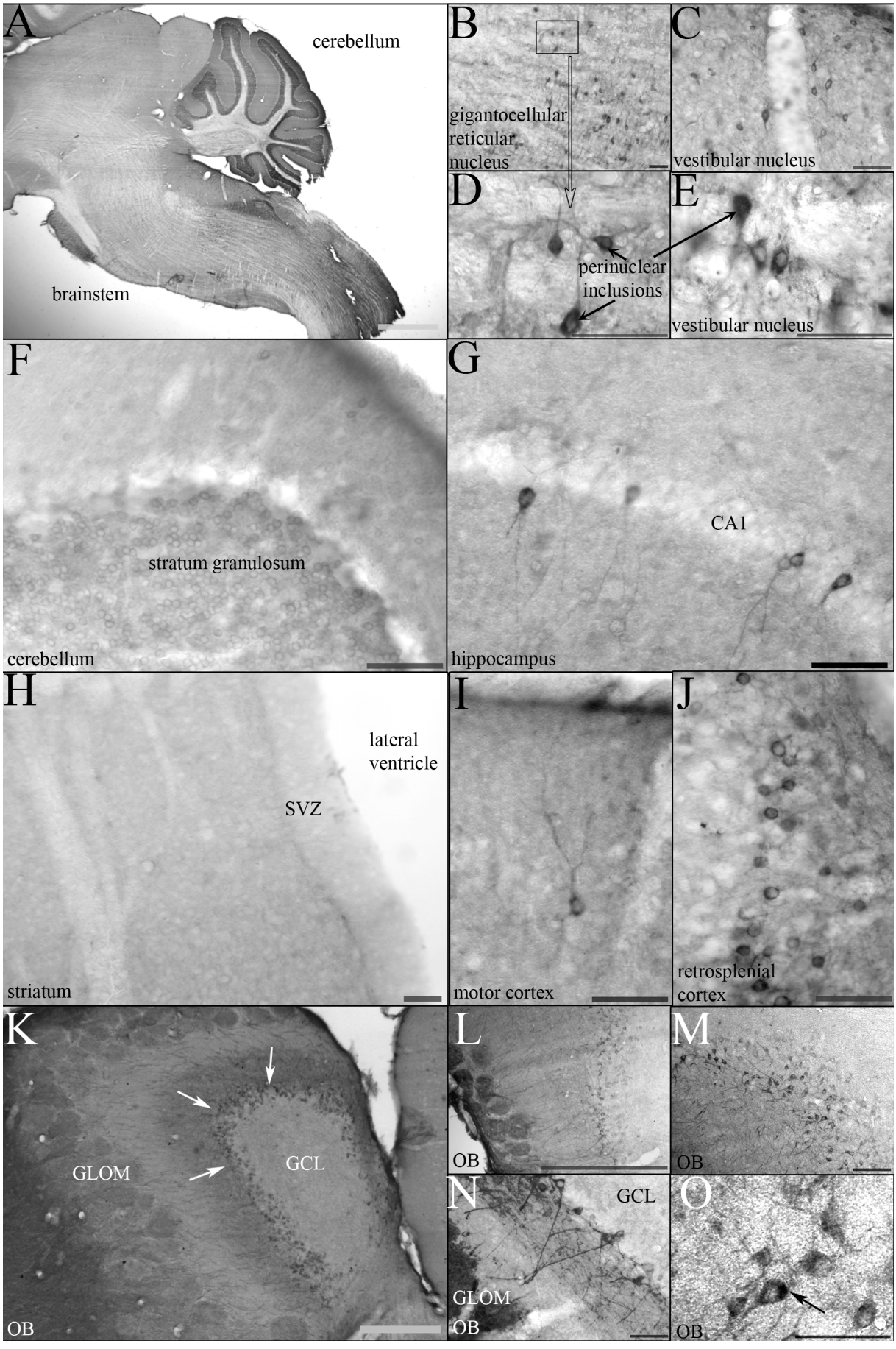
Figure 6. In the **optical fractionator counting method**, the anatomical region is first outlined (yellow) and then overlaid by a 300x300µm² grid (grey lines). Into each rectangle, a counting frame of 30x30µm² is spaced bordered by two exclusion lines (red) and two inclusion lines (green).

D. Results

1. Expression of transgenic and endogenous torsinA

V5-antibody detected transgenic torsinA in both experimental groups hWT and hMT. In hWT, immunoreactivity for transgenic human wildtype torsinA was found throughout the mouse brain. All positive cells showed a cytoplasmatic signal and no staining of the nucleus. There was a range from weakly positive to strongly positive cells. Staining was most intense in the hindbrain and the cerebellum (Fig. 7A). In the hindbrain, several nuclei contained V5-positive cells including the gigantocellular reticular nucleus (Fig. 7B), vestibular nuclei (Fig. 7C) and pontine nuclei. At highest magnification, inclusion-like bodies were detectable (Fig. 7D, E). Most positive cells within the brainstem had processes and a neuron-like shape. In the cerebellum, cells of the stratum granulosum without processes were strongly positive whereas almost no positive cells were seen in the Purkinje cell layer and the molecular layer (Fig. 7F). No positive cells were detected in the midbrain (substantia nigra, inferior and superior colliculus). The hippocampus sparsely contained positive cells in the stratum granulosum of CA1 to CA3 – but not in the DG – all of them with branches reaching into the stratum moleculare (Fig. 7G). In the thalamus, sporadic cells were seen in the corresponding nuclei. The striatum did not contain any positive cells (Fig. 7H). Throughout the cortices of hWT animals, some pyramidal cells were positive for the transgene, with an increasing number from rostral to caudal: in the motor cortex, positive cells were rare whereas they were frequently detectable in cortical visual areas and the piriform cortex (Fig. 7I, J). These cortical V5-positive cells were all located in layers 4 and 5 and most of them had a shape similar to pyramidal cells due to their triangular soma and one main apical dendrite. In the OB, V5-expressing cells were found on the outside border of the GCL. All positive cells within that layer were aligned with a polarity between the GLOM on the basal side and the GCL on the apical side. Several processes per cell were found directed towards the GLOM. They were not straightened perpendicularly towards the pial surface, but rather interweavedly going into diverse directions (Fig. 7K-M). Also, their neuron-like square somata exhibited dendritical connections between each other (Fig. 7N). No axonal or dendritical processes directed towards the GCL could be found. Similar to the hindbrain, inclusion-like bodies could be seen at highest magnification (Fig. 7O). Throughout the GCL, some V5-positive cells without processes were observed widespread in a low density without gathering. In summary, most prominent expression of the human wildtype transgene was detected in the brainstem and the cerebellum. Regarding the neurogenic regions, high expression was found in the OB only.

Transgenic human wildtype torsinA (V5-antibody)



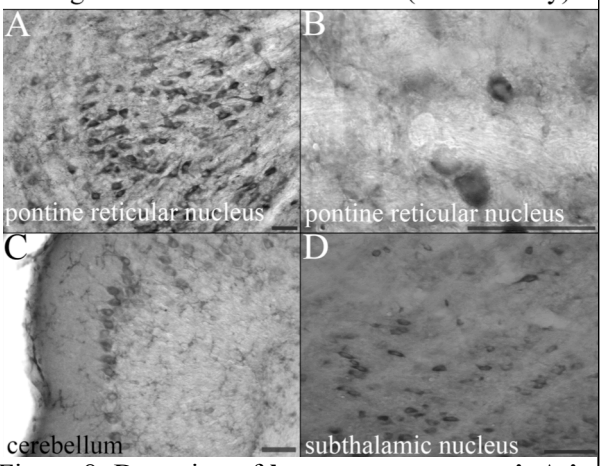


Figure 8. Detection of **human mutant torsinA in hMT mice**. Pontine reticular nucleus (A) contained many immunoreactive cells with some of them showing perinuclear inclusions (B). Cerebellar Purkinje cells (C) were also positive as well as cells of the subthalamic nucleus (D). Scale bars: 50µm.

In hMT animals overexpressing human mutant torsinA, expression was much lower compared to hWT mice – V5-positive cells could be detected in a few distinct brain areas only when using an elongated incubation time of primary antibody (see methods chapter). Similar to hWT animals, expression could also be demonstrated in the brainstem and in the cerebellum. In the brainstem, clusters of positive cells were found in the pontine nuclei exhibiting inclusion-like bodies (Fig. 8A, B). In addition, positive cells were present in the cerebellar Purkinje layer (Fig. 8C) and in the mesencephalic subthalamic nucleus (Fig. 8D). In all other areas including cortex, hippocampus and OB, no positive signal could be obtained. Altogether, in hMT animals, human mutant transgene staining was relatively low and detected mainly in brainstem, cerebellum and mesencephalon, but not in relation to the neurogenic zones.

In summary, V5-antibody revealed high expression in brains of hWT animals, low expression in brains of hMT animals and no expression in the transgene-negative control group (negative control). This is consistent with the reported levels of expression in those lines: in hMT brains, twofold expression of transgenic human mutant torsinA compared to endogenous torsinA had been measured on the protein level whereas in hWT brains, protein levels of the transgene had been sixfold compared to endogenous protein levels of torsinA (Grundmann et al. 2007).

Anti-torsinA antibody D-M2A8 was used to detect endogenous torsinA. It has also been reported to label both human mutant and human wildtype TorsinA *in vitro* (Hewett et al. 2003; Hewett et al. 2006) and *in vivo*, but not TorsinB (Hewett et al. 2004). In control animals, this

Figure 7 (*previous page*). Summary of the detection sites of **human wildtype torsinA in hWT mice**: (A) Hindbrain and cerebellum showed most prominent staining, e.g. in the gigantocellular reticular nucleus (B) and the vestibular nucleus (C) where some of the positive cells also showed perinuclear inclu-sion-like bodies (D and E, respectively). In the cerebellum, cells of the stratum granulosum primarily showed the transgene (F). In the hippocampus, few cells of the CA1 region were found positive (G), whereas transgene was not detected in the striatum (H). Cortical layers 4 and 5 contained positive cells, few in frontal areas like the motor cortex region (I) and more in caudal areas like the retrosplenial cortex (J). In the olfactory bulb, positive cells surrounded the granular cell layer, extending dendrites towards the glomerular cell layer (K - N). Here also, inclusion-like bodies were observed (O). Bars represent 50µm except for A, K and L (1mm).

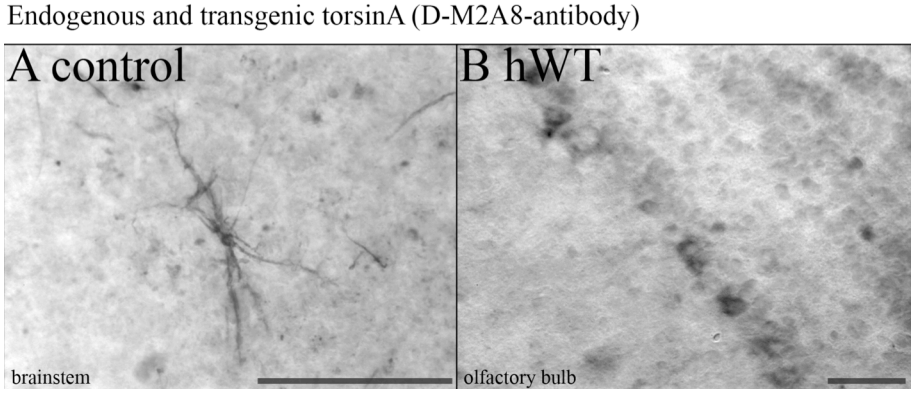


Figure 9. **Antibody D-M2A8 detects both endogenous and transgenic torsinA.** Despite high antibody titers and tissue pretreatments, signals were low in all lines. (A) TorsinA-positive cell detected in the brainstem of a control mouse. (B) Some torsinA-positive cells surrounding the granular cell layer of the olfactory bulb similar as in Fig. 7N. Scale bars: 50µm.

antibody shows the presence of endogenous torsinA. Weak immunoreactivity was found in neurons all over the mouse brain, staining the cytoplasm and the processes except for the nuclei (Fig. 9A). In hWT animals, where this antibody also recognizes transgenic protein in addition to endogenous torsinA, the distribution appeared to be more intense and more neurons were stained. Presence of the transgene within the OB surrounding the GCL could be demonstrated (Fig. 9B). In hMT animals, torsinA signal had a medium intensity and the same distribution as in control animals – here, the transgene could probably not be clearly stained due to its relatively low expression.

In summary, localization of both endogenous torsinA and transgenic human wildtype or transgenic human mutant torsinA was confirmed by DAB-immunoperoxidase stainings as previously reported (Fig. 10). Despite of its limited functionality, anti-torsinA antibody confirmed that overall torsinA levels are higher in the transgenic animal lines compared to control animals which show a normal distribution of torsinA within the mouse brain.

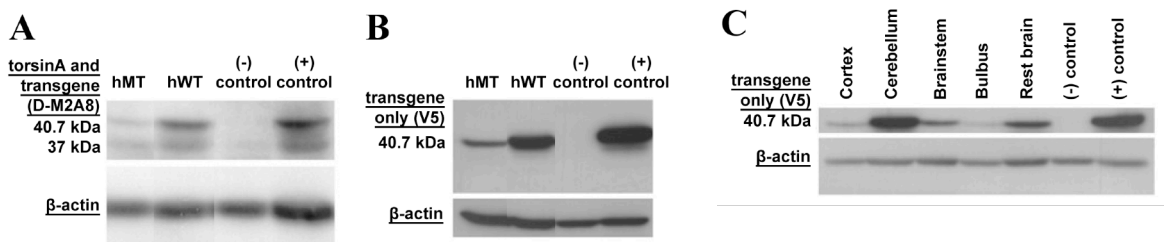


Figure 10. Western blot analyses conducted by Grundmann et al. (2007). (A) While in control animals, only endogenous torsinA is present (37 kDa), D-M2A8 antibody also detects the transgene (40.7 kDa) in the transgenic lines – at low levels in hMT and at higher levels in hWT. (B) V5-antibody specifically recognizing the transgene confirms a relatively high expression level in hWT as compared to hMT. (C) In homogenates of different brain regions of hWT mice, high transgene expression is present in cerebellum, brainstem, cortex and the OB (compare Fig. 7).

2. No colocalization of transgene and neuroblasts

In order to further approach the influence of transgenic human mutant and human wildtype torsinA on adult neurogenesis of the experimental mice, confocal laser scanning microscopy was used to evaluate colocalization of the transgene (identified by the V5-tag) and DCX-expressing cells (neuroblasts). In the control group animals, V5-antigen was not detected. DCX immunoreactivity was similar to previous reports in all groups (Brown et al. 2003b; Winner et al. 2004): In the hippocampus, DCX-positive cells were localized in the SGZ of the DG (Fig. 11A). Different morphological appearances of early and mature DCX-positive cells could also be distinguished. Early DCX-positive cells are located in the subgranular zone in clusters and have short processes parallel to the granule cell layer. Mature DCX-positive cells are characterized by localization within the granule cell layer and by long processes oriented towards or into the molecular layer. Also in the SVZ/OB neurogenic regions, DCX cells were similar to controls. In the SVZ, DCX-expressing cells had a round cell body with two or no processes and were lying close next to each other. In the RMS, their shape was elongated with long processes forming chain-like structures typically for migratory neuroblasts (Fig. 11B). In the OB, cells in the GCL extended neurites towards the mitral and glomerular cell layers (Fig. 11C) and cells within the GLOM had processes between the glomeruli (Fig. 11D). Additionally, few DCX-positive cells were localized in the striatum (see below).

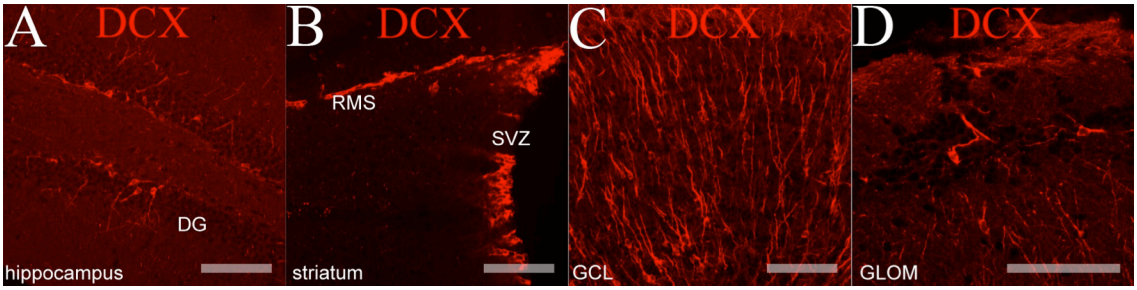


Figure 11. In control mice, **DCX immunoreactivity** occured in the dentate gyrus of the hippocampus (A), in the subventricular zone and the rostral migratory stream (B) and in the granular cell layer (C) and the glomerular cell layer (D) of the olfactory bulb. No differences were seen between controls and the two experimental groups. Scale bars: 100µm.

In hWT animals, V5-immunoreactivity was found in accordance with the distribution in the DAB-stainings: Brainstem (Fig. 12A, B) and cerebellum (Fig. 12C) showed many V5-positive cells; single positive cells were found in the hippocampal CA1 region (Fig. 12D) and in the cortex. Besides, perigranular expression of the transgene was confirmed in the OB. In the hippocampus, V5-immunoreactive cells of the CA1 had no visible contact with DCX-positive cells. In the SVZ and RMS, no colocalization was found due to the absence of V5-positive cells. In the GCL of the OB, V5-positive cells were sometimes found in proximity to the cell bodies of DCX-positive cells of the GCL. Additionally, an abundance of DCX-

positive processes reaching towards the GLOM crossed the layer of V5-positive cells. However, no colocalization of V5 and DCX was found (Fig. 13).

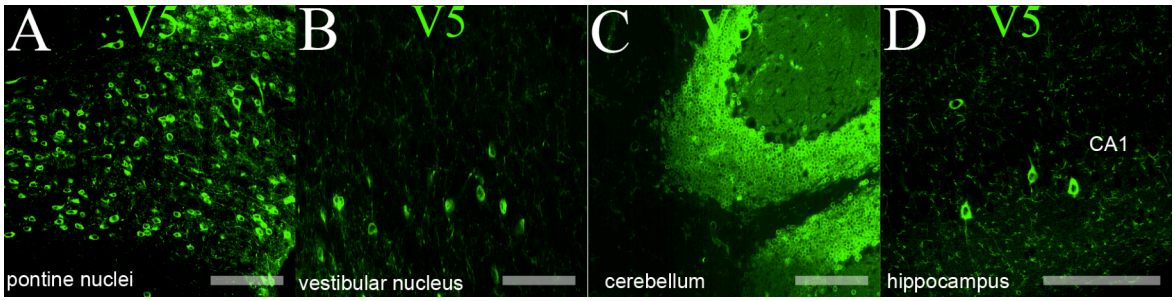


Figure 12. **Immunofluorescence of the transgene in hWT animals** was consistent with DAB stainings, e.g. in pontine nuclei (A), vestibular nucleus (B), cerebellar granule cell layer (C) and CA1 region of the hippocampus (D). Scale bars: 100µm.

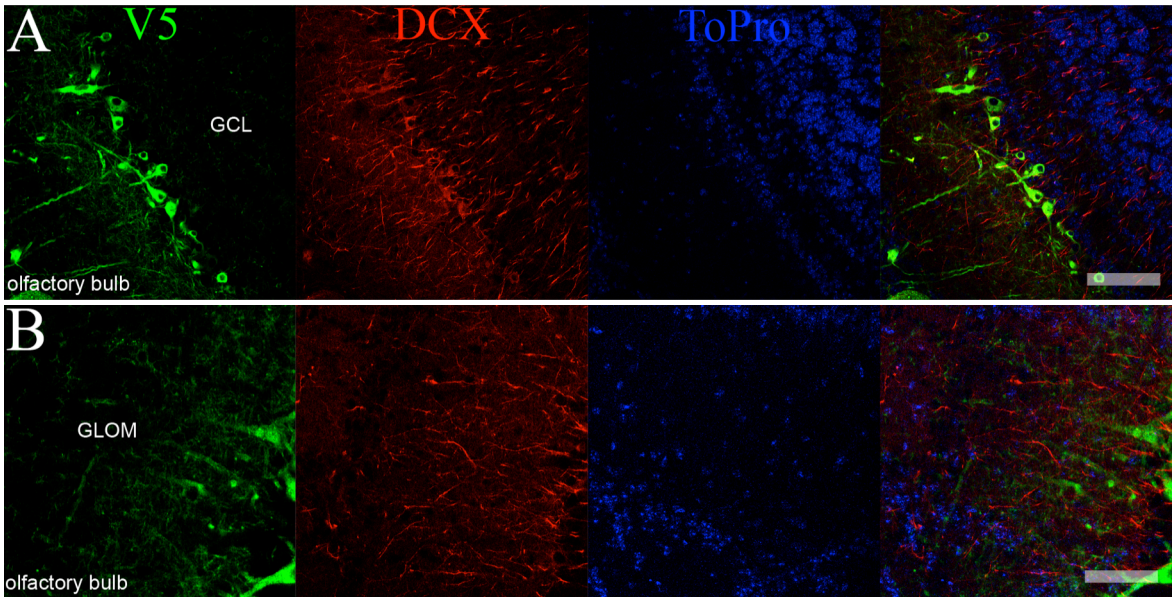


Figure 13. Colocalization studies revealing **no coexpression of transgene and the early neuronal marker DCX in hWT mice**. (A) Transgene-expressing cells around the granule cell layer of the olfactory bulb detected by V5-antibody do not colocalize with doublecortin (DCX), but are enclosed by doublecortin-positive processes. (B) In the glomerular cell layer (left half of pictures), doublecortin-positive fibers crossing the transgene-positive cells ended within the glomeruli. Scale bars: 100µm.

In hMT animals, V5 transgene was not detected within the neurogenic regions as found in the DAB-sections, thus, no colocalization could be demonstrated.

In summary, the distribution of DCX expressing cells was similar in all groups and no colocalization of the DYT1 transgene and DCX could be found in the two transgenic groups. The only site where transgene and DCX-positive cells were in close proximity was the GCL of hWT animals.

3. Normal patterns of BrdU, PCNA and DCX in brains of transgenic animals

To label newly generated cells, animals aged 5 months received daily injections of BrdU for 4 days and were sacrifized 28 days later. In all groups, BrdU immunoreactivity was predominantly found in the GCL of the OB, in the DG of the hippocampus (Fig. 14A-C) and in the SVZ and RMS. Besides, BrdU-positive cells were found at low density all over the brain, representing glial or endothelial cells that have undergone cell division.

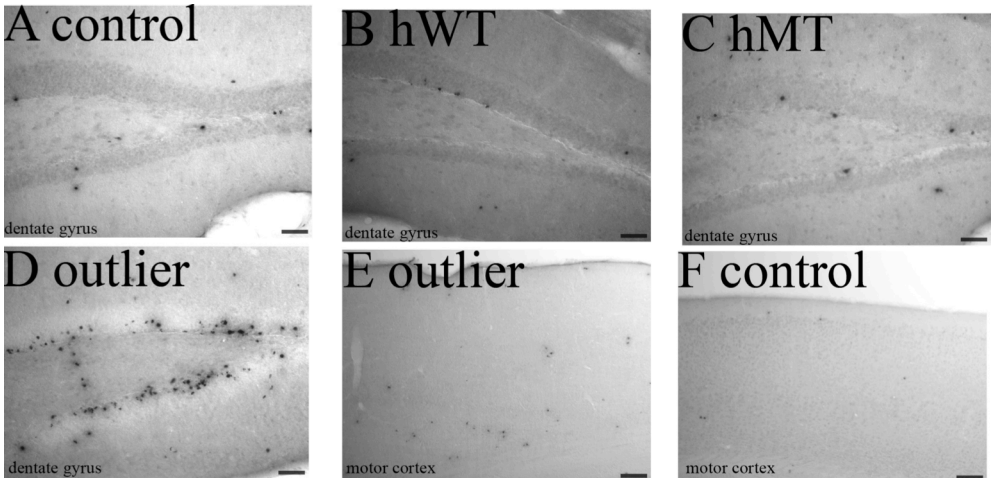


Figure 14. **BrdU staining of the dentate gyrus** reveals no differences between (A) control, (B) hWT and (C) hMT animals. One animal of the hMT group was excluded from analyses because of abnormally increased and abnormally located BrdU-labeled cells in the dentate gyrus (D) and the cortex (E) when compared to controls (F). Scale bar = 50µm.

PCNA is expressed in early G_1- and in the S-phase of the cell cycle and used to determine the rate of cell proliferation within the neurogenic zones (Hall et al. 1990). PCNA staining patterns were similar in all groups. High immunoreacitivity was found in the neurogenic zones. Positive cells were located at lower density along the RMS and at its entry into the GCL. Besides, some PCNA-positive cells were dispersed all over the brain.

In one animal of the hMT group, numbers of BrdU-positive cells were severalfold increased, especially in the cortical areas and the hippocampus (Fig. 14D-F). Hippocampal cell numbers of this animal exceeded the mean cell count of the other animals of this group by 39 standard deviations whereas olfactory bulb counts were within the 95% confidence intervals. Repeated immunostaining obtained the same results. Therefore, we hypothesized pathological changes in the brain of this animal, e.g. epileptic seizures (Parent et al. 1997; Couillard-Despres et al. 2005) and excluded it from further analyses.

No abnormal migration of DCX-positive cells: For the investigation of the striatum, the distribution of striatal DCX-immunoreactivity was analyzed in sagittal and coronal sections. In all groups, DCX-labeled cells were rarely found at a maximum of 3 cells per section, mostly

close to the SVZ (Fig. 15A,B). All cells had a mature appearance with one or more processes extended into different directions.

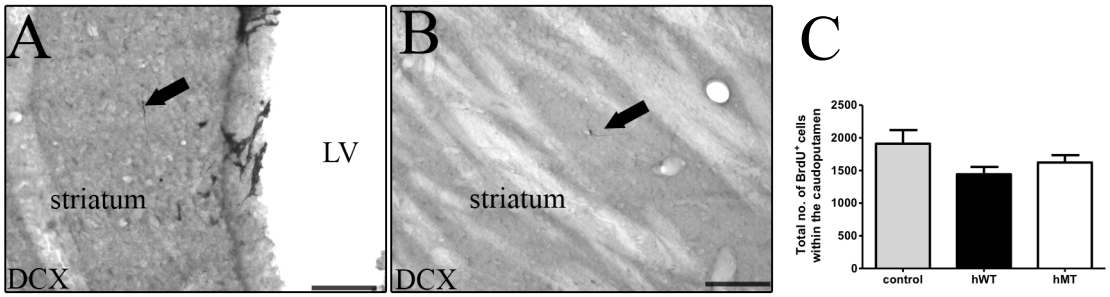


Figure 15. **Doublecortin-positive cells of the striatum** were mostly found near the subventricular zone (A), rarely in more rostral parts (B) with no obvious differences between groups. The number of striatal BrdU-positive cells did not differ between groups (C). *LV* lateral ventricle. Scale bar = 100µm.

Moreover, we quantified striatal BrdU-positive cells (with the following borders: lateral ventricle caudally, corpus callosum dorso-rostrally and a sagittal straight line through the anterior commissure ventrally) in order to detect proliferative changes. No change was found between the three groups (Fig. 15C).

4. Unchanged proliferation, survival and differentiation of adult newborn neurons in both transgenic groups

Unchanged hippocampal proliferation and survival: PCNA- and BrdU-positive cells were counted to compare cell proliferation and survival of the newborn cells, respectively, between the three groups (Table 6). Estimated volumes were compared to check if the transgene had any morphological impact on the dentate gyrus. The mean estimated volume of the dentate gyrus was 0.10mm³ in all groups without significant differences (Fig. 16A). Statistical analysis of the number of BrdU-positive cells in the dentate gyrus showed no significant difference between the groups (Fig. 16B). There was also no difference in PCNA cell counts (Fig. 16C). This means that none of the two transgenes has an influence on adult hippocampal neurogenesis, neither on cell proliferation nor on cell survival.

Region	control	hWT	hMT
Dentate gyrus			
Volume (µm³)	0.1022 ± 0.002894	0.1061 ± 0.004004	0.1011 ± 0.002594
BrdU-positive cells	514.5 ± 67.41	593.1 ± 66.69	551.1 ± 22.39
PCNA-positive cells	634.5 ± 127.1	535.7 ± 59.23	570.9 ± 68.38
Granular cell layer (OB)			
Volume (µm³)	1.545 ± 0.08600	1.517 ± 0.05201	1.531 ± 0.04356
BrdU-positive cells	46500 ± 2689	49710 ± 4162	52030 ± 3958
Ratio $NeuN^+/BrdU^+$ cells	0.8538 ± 0.04589	0.8045 ± 0.00990	0.8908 ± 0.01902
Newborn neurons	39970 ± 4078	40160 ± 3696	43720 ± 2454
Glomerular cell layer (OB)			
Volume (µm³)	0.5820 ± 0.05155	0.5757 ± 0.08131	0.6240 ± 0.05153
BrdU-positive cells	3979 ± 415.7	3585 ± 562.3	3979 ± 415.7

Table6. Stereological quantification of neurogenesis.in the animal models. Values are indicated ± standard error of the mean. There were no significant differences between groups.

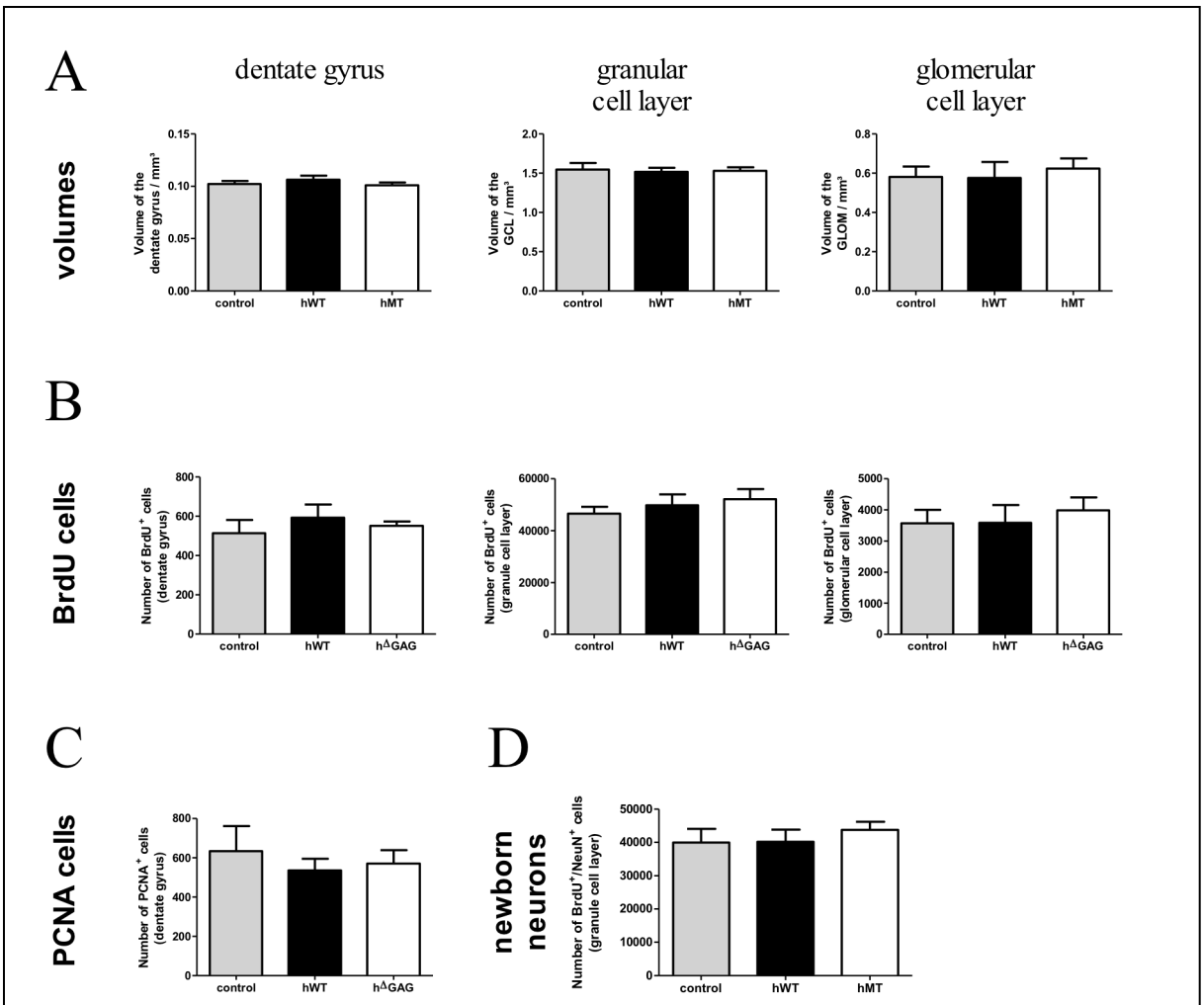


Figure 16. **Stereological quantifications.** Both transgenic lines did not differ from controls regarding (A) volumes, (B) numbers of BrdU-positive cells and (C) numbers of PCNA-positive cells in the dentate gyrus and the granular and glomerular cell layer of the olfactory bulb. (D) There was also no difference in newborn neurons of the GCL.

Unchanged olfactory bulb neurogenesis: In the OB, comparison of the underlying volumes of the GCL and the GLOM revealed no difference (Table 6, Fig. 16A). Again, no influence of the transgene on the number of BrdU-labeled cells was detected, neither for the GCL nor for the GLOM (Fig. 9B). With no difference in the numbers of BrdU-positive cells of the OB given, we proceeded without analyzing PCNA in the SVZ/OB because altered proliferation would be expected cause altered survival counts. Even if there was altered proliferation, a functional impact would be unlikely due to unchanged survival. In order to ultimately exclude differences in the generation of newborn *neurons*, we analyzed differentiation of BrdU-positive cells in the GCL by colocalization with the mature neuronal marker NeuN. Ratios of neuronal differentiation did not differ between groups (Table 6) and resulting estimated numbers of newborn neurons also showed no change (Table 6, Fig. 16D).

E. Discussion

In the present study, the effect of overexpression of human wildtype and human mutant torsinA on adult neurogenesis was examined in two transgenic mouse models. Partial presence of the transgenes within the neurogenic zones was shown, but no localization within neuroblasts could be found. Quantitative analyses of BrdU and PCNA immunohistochemistry revealed no difference between transgenic and control groups concerning the number of newly generated cells and newborn neurons. This indicates that human wildtype and human mutant torsinA do no alter adult neurogenesis in these models of DYT1 primary torsion dystonia.

1. Methods to quantify adult neurogenesis

Our results of adult neurogenesis in the two transgenic mouse models are based on the use of established methods to determine adult neurogenesis in rodents.

Antibodies: The antibodies used for immunohistochemistry (directed against torsinA, V5-tag and DCX) have all been characterized and are specific for their antigens. The DCX-antibody revealed a normal staining pattern. Both antibodies against torsinA and V5-tag only recognized the antigen when using antigen enhancement methods. We could show endogenous and transgenic torsinA in different brain regions. TorsinA protein revealed by anti-torsinA antibody was present in neurons all over the brains of control mice. Staining was more intense in the high expressing hWT line and intermediate in the low expressing hMT line. V5-tagged transgenic torsinA could be detected in all areas described by Grundmann et al. (2007) except for the striatum. Therefore, tissue preparation methods might have reduced the sensitivity of the torsinA and V5-tag antibodies.

BrdU labeling: Out of the number of neuroblasts that are generated during the 4 days of BrdU injection, only a limited amount will survive as a new part of the neurogenic target region. All other newly generated cells die during the first 4 weeks after BrdU injection (Kempermann et al. 2003). Thereafter, the rate of BrdU-labeled neurons remains basically unchanged at least for 1.5 years as it has been shown for the rat OB and the mouse hippocampus (Winner et al. 2002; Kempermann et al. 2003). Consequently, neurons in our study that were BrdU-labeled 28 days after the infusion are most probably integrated and mostly represent new neurons within the neurogenic destination zones. Recent reports found that neural stem cells retain the

same strands of DNA during all divisions [immortal strand hypothesis (Karpowicz et al. 2005)]. Therefore, BrdU might not label the whole population of cycling stem cells, but will label deriving precursor cells and neuroblasts. As BrdU has been characterized intensively regarding its use in adult neurogenesis research, the injection paradigm that we used is a valid tool to determine the rate of survival of new neurons in the neurogenic zones.

2. Conclusions for the pathophysiology of the mouse models

No cell-autonomous effects of transgenic torsinA on neural precursors: As first aim, we investigated expression patterns of the transgenes. In both transgenic lines, transgenic torsinA was not expressed in neuroblasts of the adult brain, neither within the SGZ or the DG nor within the SVZ or the OB. As the transgenes were not present in any cells of the two niches of active adult neural stem cells (SVZ and SGZ), we infer that they are also not expressed in neural stem cells. In the OB of the hWT group, cells at the outer margin of the GCL were positive for the hWT torsinA. These cells were most likely mitral cells because they had primary dendrites directed into the GLOM and secondary dendrites to other cells of this layer. Accordingly, no colocalization was found with the neuroblast marker DCX, and DCX-positive processes of immature granule cells were in contact with the transgene-expressing cells. As the transgenes are not expressed in neural stem cells, neuroblasts or newly generated mature neurons, they are unlikely to exert cell-autonomous effects on adult neurogenesis in both animal models. We therefore speculate that potential interactions between torsinA and adult neurogenesis are cell non-autonomous.

No migration of newborn neurons into the striatum: We also suspected that the migration of newborn cells could be redirected in transgenic mice. We examined the caudate putamen part of the striatum particularly carefully for presence of DCX- and BrdU-labeled cells because the striatum of hWT animals had been shown to be affected by the transgene in matters of neuropathology, white matter circuit changes, behavioral phenotype and metabolism of dopamine and serotonine (Grundmann et al. 2007). Besides, abnormally localized DCX-positive cells in the striatum were speculated in advance (Grundmann, personal communication).
The observation that some DCX-positive cells are also found in the striatum is not a new one. Rather than being neuroblasts, these striatal DCX-positive cells have been associated – in non-pathologic wildtype animals – with non-migrating neurons undergoing plasticity in form of synaptic reorganization (Nacher et al. 2001).

hMT animals do not show neurochemical changes in the striatum, but striatal dopamine and serotonin levels are decreased in hWT animals (Grundmann et al. 2007). Experimental striatal dopamine-depletion by 6-OHDA lesion does not lead to increased striatal DCX-positive cells (Winner et al. 2008a). Importantly, striatal DCX expression and striatal BrdU incorporation did not vary between groups excluding a redirected migration of neuroblasts into the SVZ-adjacent striatum. This means also that we could not find signs of enhanced striatal plasticity reflected by increased DCX expression. Also in other motor regions of the brain, patterns of BrdU, PCNA and DCX immunoreactivity did not differ from controls. In particular, no excessive cell numbers were found in motor cortex, SNc and cerebellum. We conclude that altered plasticity in the basal ganglia circuits of DYT1 mice is not due to the integration of new neurons.

Aggregation per se does not decrease adult neurogenesis: As we found torsinA-related inclusion bodies in mitral cells of the OB in close relation to the GCL and the GLOM, we expected an effect of torsinA at the integration site of newborn granule cells. Against our own anticipation, torsinA-related inclusions, however, did not affect the survival of newly born cells despite its accumulation in the local microenvironment. In the mouse model of Marxreiter et al. (2009), transgenic human α-synuclein was overexpressed in mature granule cells of the OB. In this model, inclusions led to increased cell death of newly generated neurons. Therefore, we hypothesize that α-synuclein expression in newborn neurons may lead to cell-autonomous effects at their integration site. The lack of torsinA-associated cell-non-autonomous effects may due to its lack of expression within mature newborn neurons compared with α-synuclein.

Influence of altered neurotransmitter levels: The fact that we could not detect an influence of the transgenes on adult neurogenesis is also surprising since both transgenic groups show alterations of neurotransmitters that have been reported to modulate adult neurogenesis.
Striatal dopamine and serotonin levels in hWT animals are significantly decreased as well as the dopamine metabolite HVA within the brainstem. Accordingly, hWT animals show a hypoactive phenotype in behavioral tests (Grundmann et al. 2007). In hMT animals, the dopamine metabolite DOPAC as well as serotonin and its metabolite 5-HIAA were increased in the brainstem. Possibly as a consequence of increased serotonin, they were hyperactive (Grundmann et al. 2007). In the majority of other murine animal models of DYT1 dystonia published so far, levels of striatal dopamine and its metabolites were also altered (Dang et al.

2005; Sharma et al. 2005; Shashidharan et al. 2005; Dang et al. 2006; Balcioglu et al. 2007; Zhao et al. 2008b). Both dopamine and serotonin have repeatedly been shown to promote adult neurogenesis (Baker et al. 2004; Banasr et al. 2004; Hoglinger et al. 2004; Winner et al. 2006). Changes in proliferation and survival are revealed most easily in young animals at the age of about 2 months due to the age-dependent decline of adult neurogenesis (Kuhn et al. 1996; Tropepe et al. 1997). Nevertheless, we chose 5 months old animals for our analyses because in both lines, behavioral and neurochemical changes could only be detected from this age on (Grundmann et al. 2007) which may have obscured minor changes of adult neurogenesis. Therefore, the relatively small changes in transmitter levels (that did not exceed 25%) probably were not effective enough to produce a detectable change of adult neurogenesis (Wichmann 2008). Decreased striatal dopamine levels could also have compensated the effects of increased serotonin levels.

Two other features of the mouse models (neuropathological inclusions and altered white matter connectivity) occurred independent of the age of the transgenic animals. Therefore, an effect of these on adult neurogenesis is unlikely.

3. The DYT1 mouse model of dystonia

To be able to confer findings in animal models to the human disease, it is important to note that animals bear important differences in lifespan, physiology and disease etiology. In animal models, one or few cardinal features of the human disorder are replicated, but it is rarely possible to produce an identical phenotype. Rodent animal models are widely used in research because they are also mammalian, they show a comparable anatomy, are easy to handle, can be modified genetically and have a relatively short reproductive cycle period. However, their limited lifespan makes it difficult to model chronic diseases of the CNS that develop over several years.

Comparison to other DYT1 models: In the knock-in, knock-down and knock-out dystonia mouse models, histological results predominated the phenotype (Dang et al. 2005; Goodchild et al. 2005; Dang et al. 2006). Models transgenic for human mutant torsinA in addition exhibited behavioral and neurochemical alterations in dopamine metabolism (Shashidharan et al. 2005; Pisani et al. 2006; Balcioglu et al. 2007; Grundmann et al. 2007; Zhao et al. 2008b).

Individual results concerning behavioral alterations and neurochemical changes differ between different DYT1 transgenic models. Differences in genetic background, constructs used

for transfection and age at the time of analysis have been discussed as possible reasons for inhomogeneities between the models (Wichmann 2008; Zhao et al. 2008b).
For example, Shashidharan et al. (2005) observed a dystonic movement phenotype in 40% of all transgenic mice. This limited penetrance unlikely corresponds to the 30% penetrance of DYT1 in humans, but rather resulted from insufficient genetic uniformity since the authors investigated F1 offspring only. In all the other models, reduced penetrance of the phenotypes could not be replicated, possibly because the putative second hit (environmental or genetic) is missing that human mutation carriers need in order to develop symptoms.
We assume that the two models used in our study are appropriate because they are derived from a cause known to cause PTD in humans (etiologic validity) and because they deliver reproducible results that are partially in line with earlier models (reliability). However, no obvious motor syndrome of involuntary muscle contractions is present in the animals (Jinnah et al. 2005).

Time of investigation: In human carriers of the DYT1 mutation, PTD has an onset between the age of 5 to 25 years in one third of all cases while the remaining two thirds are unaffected throughout life. Compared to classical neurodegenerative disorders, this is early, but it might be too late compared to the 2 to 3 years lifespan of a laboratory mouse. Therefore, the effects of the genetic modification are often multiplied by expression of a transgene under a strong promoter. Consistently, the alterations in DYT1 knock-in, knock-down and knock-out mice are less pronounced than in the available transgenic DYT1 models.

Spatial expression pattern of the transgenes: Endogenous torsinA protein is expressed weakly in cells of all grey matter regions and highly in SNc, cerebellum and striatum (Shashidharan et al. 2000; Konakova et al. 2001; Konakova and Pulst 2001; Augood et al. 2003). In the CNS, the murine prion protein promoter drives mRNA expression in neurons of all brain areas and to a lower extent in glial cells (Kretzschmar et al. 1986; Moser et al. 1995; Borchelt et al. 1996). Endogenous mouse prion protein was found ubiquitously in all subpopulations of neurons and some glial cells (Laine et al. 2001). Others reported it to be restricted to GABAergic neurons of the cortex and the cerebellum only, highlighting the important role of transcriptional, post-transcriptional and post-translational regulation of the prp-protein (Ford et al. 2002). In a study that expressed α-synuclein under control of the murine prp-promoter, CNS protein expression was most abundant in cortex, hippocampus, colliculi and cerebellum (Maskri et al. 2004). In our study, however, expression occurred strongest in areas

of the brainstem and the cerebellum. This difference might be attributed to the different lengths of the prp-promoters that were used as the mouse prion protein exhibits promoter activity at least up to exon 2 (Baybutt and Manson 1997). Expression or overexpression of torsinA might also be impaired in some brain areas due to post-transcriptional regulations like low translation efficiency, impaired protein trafficking or short half-life of the transgene. A possible reason for the disparity of distributions of endogenous and transgenic torsinA in our study could be the fact that human and not mouse torsinA was used as transgene and is therefore processed differently. Moreover, the V5-tag of the transgenes can also influence transcription or stability of the construct. The immunohistochemical localization of transgenic human torsinA in turn was similar to that observed in the transgenic mice by Shashidharan et al. (2005) who also expressed human mutant torsinA, but under the control of the neuronal NSE-promoter. In both of these models, intracellular aggregations were found in nuclei of the brainstem. Similar inclusion bodies were reported in human PTD patients, also restricted to their brainstems (McNaught et al. 2004). In summary, the expression pattern of transgenic torsinA in the mouse models that we used is consistent with another mouse model. In addition, the promoter is able to replicate the brainstem morphology of PTD patients.

Temporal expression patterns of the transgenes: As torsinA is thought to exert its functions predominantly during neural development, it is important to evaluate how expression patterns of endogenous and transgenic torsinA overlap temporally.

A study in humans showed that the expression of human endogenous torsinA starts within most brain regions at the postnatal age of 4 weeks with an abrupt onset; it is most prominently expressed around 6 weeks of age and declines down during childhood to a supposedly lifelong plateau of expression (Siegert et al. 2005). Hence, the time of peak expression does not overlap with the morphological development of brain structures, but it is paralleled by the period of synaptogenesis, synaptic remodeling and process elimination. In accordance with the developmental expression of torsinA in humans, mouse and rat torsinA expression peaks from embryonic day 15 to postnatal day 14 (Xiao et al. 2004; Vasudevan et al. 2006).

In contrast, mouse prp-promoter is not active in embryonic neuronal precursor cells of mice, but is upregulated in neurons upon complete differentiation (Tremblay et al. 2007). Another study showed that in adult mice, prion protein is expressed highly in mature neurons and also immediately adjacent to the SVZ, but not in mitotically active cells (Steele et al. 2006).

This is compatible with our observation that neither of the transgenes was expressed in neural precursor cells or neuroblasts. Consequently, we assume that there is a mismatch between

endogenous and transgenic torsinA expression concerning their developmental expression patterns: endogenous torsinA appears early during neuronal migration and synaptogenesis and declines thereafter whereas the prp-promoter is specifically active in differentiated neurons and maintains high expression throughout life.

Thus, our animal models might not have been able to reflect a cell-autonomous effect of DYT1 on adult neurogenesis. It has not been shown if endogenous torsinA is expressed in *adult* neural precursor cells because there are only studies about embryonic expression (Vasudevan et al. 2006). There are complex transitions between embryonic/perinatal and adult neurogenesis, so results from embryonic studies cannot be extrapolated to the adult situation (Altman and Bayer 1990; D'Amour and Gage 2003). Therefore, future studies should first characterize the expression of endogenous torsinA in adult neural stem and progenitor cells.

Conclusions for adult neurogenesis in DYT1 patients: As demonstrated above, animal model in this study only partially reflects DYT1-related dystonia in humans. The model still provides a good means to investigate certain aspects of the disease. The transgenic models that we used replicate the findings of intraneuronal inclusion bodies in the brainstem of DYT1 patients. Thus, we conclude that these inclusions likely do not affect adult neurogenesis. Besides, there was no evidence for reactive alterations in adult neurogenesis due to basal ganglia pathology.

4. Conclusions for primary dystonias in humans

Subcellular forms of neuronal development and plasticity involved in dystonia: Neither hereditary nor sporadic forms of PTD show a phenotype from birth on, but have an onset during lifetime. Signs predicting if and at which time the disease will evolve are not known up to now (Bressman 2004).

TorsinA, on the contrary, is thought to exert its functions predominantly during brain development: Homozygous knock-out of wildtype torsinA results in a lethal phenotype (Goodchild et al. 2005). Besides, torsinA expression in rodents and humans is highest during the phases of cortical synaptogenesis, synaptic remodeling and process elimination (Xiao et al. 2004; Siegert et al. 2005; Vasudevan et al. 2006). In human cortical brain areas, this span extends from 6 to 16 years of age (Huttenlocher 1979; Huttenlocher 1990; Andersen 2003) coinciding with the temporal window of DYT1 PTD onset. Before the PTD onset, embryonic neurogenesis has ceased in most areas of the brain – consistent with the absence of gross morphological

abnormalities in DYT1 patients and dystonia patients in general. Therefore, it seems plausible that the pathophysiology of dystonias is due to abnormal connectivity between neurons rather than due to alterations in the embryonic generation of neurons.
The development of sporadic, adult-onset primary dystonias, especially task-specific dystonias like writer's cramp, has been related to excessive sensory stimulation or repetition of motor tasks (Quartarone et al. 2006). Intense training of motor skills during writing or when playing an instrument leads to somatotopic reorganization of activated sensorimotor areas both in cortex and basal ganglia. This reorganization is thought to be mediated by synaptic changes resulting in a decrease of inhibitory inputs to the affected motor areas (Watson 2006).

Imbalances of striatal dopaminergic metabolism in dystonia and Parkinson's: The role of striatal dopamine metabolism in the development of dystonias is supported by many findings. For instance, striatal dopamine metabolite levels are elevated in human tissue specimen of dystonia patients (Augood et al. 2002), nuclear imaging studies showed abnormal striatal dopaminergic neurotransmission in dystonia patients (Perlmutter and Mink 2004) and dopamine receptor antagonists in schizophrenia may cause dystonic side-effects (Casey 2004). All transgenic animal models of dystonia where striatal transmitter levels have been analyzed show alterations of dopamine metabolism (Zhao et al. 2008b). In humans, gene mutations linked to dopaminergic neurotransmission, e.g. in the tyrosine hydroxylase gene or the dopamine D2 receptor gene, cause genetic forms of dystonia (Klein and Ozelius 2002). In addition, the symptom dystonia is frequently found in PD patients, as a complication of long-term dopaminergic treatment – known as "off-phase" dystonia – or as the initial symptom of the disease (Katzenschlager et al. 2002; Bruno et al. 2004; Ceballos-Baumann and Conrad 2005).
Interestingly, dopamine modulates striatal synaptic plasticity in PD (Picconi et al. 2003). Plasticity processes in humans can be examined by measuring the facilitation of motor cortex stimulation after stimulation of another cortical area or a peripheral nerve. In PD patients, these kinds of facilitation are effective only in the "on"-state which is characterized by high availability of dopamine, but not in the dopamine-lacking "off"-state (Mir et al. 2005; Ueki et al. 2006). This role of dopamine in modulating plasticity might also contribute to dystonia, and again, would not involve the generation of new neurons.
Despite similarities between PD and dystonia, no degenerative indications of neuronal loss or inflammation are found in brains of dystonia patients whereas in PD, dopaminergic degeneration in the SNc leads to motor dysfunction (Rostasy et al. 2003). In DYT1 dystonia, mutant

torsinA accumulates in the perinuclear space of neurons, but obviously is not as toxic as α-synuclein in PD.
Another important difference between PD and DYT1 dystonia is the extent of dopaminergic dysfunction. In PD, dopamine levels are substantially reduced whereas in dystonia, dopamine or its metabolites are only modestly affected (Wichmann 2008).

Symptoms related to impaired neurogenesis: In PD, HD and depression, impairments of adult neurogenesis could contribute to non-motor symptoms, particularly to the decline of hippocampus-dependent memory and to the loss of odor perception (Mirescu and Gould 2006; Ross et al. 2008). Primary dystonia, in contrast, is a mere motor disease without pronounced additional neurological or psychiatric abnormalities. Observed comorbidity of depression in dystonia is most likely secondary rather than being causally related to the DYT1 mutation (Heiman et al. 2004; Miller et al. 2007).

Perspectives: To investigate the involvement of adult neurogenesis in DYT1 dystonia patients, the effect of DYT1 overexpression or knock-down in cultured neural precursor cells on proliferation and differentiation could be examined. *In vivo*, hippocampal and SVZ cell proliferation of DYT1 brain specimen may be determined to show definite evidence of unaltered precursor proliferation in the patients. Since dystonia is related to changes in plasticity and neurons generated in adulthood provide a model to study the formation of new neuronal circuits, we also suggest to investigate qualitative integration of newborn neurons in the transgenic animals as measured by dendrite and spine numbers, for example. Finally, it would be feasible to treat transgenic animals with dopaminergic compounds in order to investigate effects on motor dysfunction and on adult neurogenesis.

F. Manuscript: *Adult neural precursor cells unaffected in animal models of DYT1 dystonia (Neuroreport, 2009)*

Adult neural precursor cells unaffected in animal models of *DYT1* dystonia

Martin Regensburger[a], Zacharias Kohl[b], Kathrin Grundmann[c], Beate Winner[a,d], Olaf Riess[c] and Jürgen Winkler[b,e]

Hereditary dystonias in humans are frequently related to a specific mutation of the *DYT1* gene that encodes torsinA. This mutation has been shown to disrupt neuronal cell migration during development. We compared adult neurogenesis, occurring in the hippocampus and the olfactory bulb, in transgenic mice overexpressing either the wild-type or mutant form of human torsinA. Neurogenesis was assessed by quantification of bromodeoxyuridine-labeled cells. Both transgenic mouse models displayed perinuclear inclusions in the brainstem and in mitral cells of the olfactory bulb, altered striatal dopamine levels, and behavioral abnormalities. However, both hippocampal and olfactory neurogenesis levels were unchanged compared with control animals. We conclude that overexpression of human wild-type or mutant torsinA does not affect the survival of adult newborn neurons. *NeuroReport* 20:1529–1533 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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[a]Department of Neurology, University of Regensburg, Regensburg, [b]Division of Molecular Neurology, University Hospital Erlangen, Erlangen, [c]Department of Medical Genetics, University of Tübingen, Tübingen, Germany, [d]Laboratory of Genetics, The Salk Institute for Biological Studies and [e]Department of Neurosciences, University of California San Diego, La Jolla, California, USA

Correspondence to Jürgen Winkler, MD, Division of Molecular Neurology, University Hospital Erlangen, Schwabachanlage 6, Erlangen 91054, Germany Tel: +49 9131 85 39324; fax: +49 9131 85 36597; e-mail: juergen.winkler@uk-erlangen.de

Martin Regensburger and Zacharias Kohl contributed equally to this study

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Introduction

Dystonias comprise a heterogeneous group of movement disorders and are characterized by sustained, involuntary muscle contractions [1]. Autosomal-dominant *DYT1* dystonia is the most common hereditary form presenting with early onset and generalized twisting movements. It is linked to a specific 3 bp-deletion (ΔGAG) in the *DYT1* gene coding for torsinA [2]. TorsinA is found in the lumen of the nuclear envelope and the contiguous endoplasmic reticulum [3], and belongs to the AAA+ (ATPase associated with a variety of cellular activities) protein superfamily [2]. TorsinA is upregulated in response to cellular stressors [3] and may function as a chaperone protein [4]. In addition, torsinA is highly expressed during development [5] and has been shown to promote neuronal migration [6]. The pathophysiology of *DYT1* dystonia has been linked to altered brain development and plasticity: affected *DYT1* patients exhibit abnormal volumes of basal ganglia subregions [7] and increased synaptic plasticity as shown by excessive excitability after repetitive transcranial magnetic stimulation [8] and by disorganization of the somatotopic representations of motor and sensory inputs to the striatum [9].

New neurons are generated not only during embryonic development, but also in the adult human brain throughout lifetime [10,11]. These neurons derive from multipotent neural stem cells residing in two neurogenic zones, the hippocampus and the subventricular zone. From the site of their birth in the neurogenic regions, adult neural precursor cells migrate and mature into functional neurons of the granule cell layer of the hippocampal dentate gyrus (DG) and the olfactory bulb (OB). Thus, studying the impact of overexpression of a protein in these regions may reveal its influence on neuronal development in the adult nervous system. Given the putative functions of torsinA during neuronal development and migration, we hypothesized that the generation of new neurons and striatal plasticity may be altered in animal models of *DYT1* dystonia.

Both transgenic mouse lines overexpressing human wild-type (hWT) or mutant (hΔGAG) torsinA under the murine prion protein (PrP) promoter similarly show perinuclear inclusions, but differ as to behavioral abnormalities and neurotransmitter levels [12]. While hWT mice are hypoactive and show decreased striatal dopamine levels compared with control animals, hΔGAG mice are hyperactive and show increased levels of dopamine. Similar findings have been observed in other transgenic models of *DYT1* dystonia [13–15]. Here, we characterized the influence of transgenic hWT or hΔGAG torsinA overexpression on adult neural stem and progenitor cell proliferation and survival in both neurogenic regions as well as in the striatum.

Methods

Animals

Mice expressing hWT torsinA ($n = 7$) or hΔGAG torsinA ($n = 7$) under control of the PrP promoter and littermate

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controls ($n = 5$) were bred from the C57Bl/6N background and genotyped as described earlier [12]. The torsinA protein expressed in transgenic hWT or hΔGAG mice was tagged C-terminally with a V5-His tag. Transgenic mice belonged to the lines exhibiting highest expression levels of the transgenic torsinA (hWT24 six-fold and hΔGAG3 two-fold greater than the level of endogenous mouse torsinA). Animals were kept in 12-h light–dark cycle and had free access to food and water. To label replicating cells, the thymidine analog bromodeoxyuridine (BrdU) was administered once daily for 4 consecutive days by intraperitoneal injection (50 mg/kg body weight) beginning at 4 months of age. Four weeks after the last BrdU injection, animals were deeply anaesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, postfixed overnight in 4% paraformaldehyde at 4°C, and stored in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4°C. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local governmental commission for animal health.

Tissue processing

On a sliding microtome in dry ice, right hemispheres of each brain were cut into 25-µm-thick sagittal sections, left hemispheres into 40-µm coronal sections. Sections were stored at 4°C in cryoprotectant solution (25% ethylene glycol, 25% glycerol in 0.1 M phosphate buffer, pH 7.4).

Immunostaining

Immunostainings were conducted as described earlier [16]. For BrdU and proliferating cell nuclear antigen (PCNA) stainings, tissue was pretreated with formamide and HCl to denature DNA. Free-floating sections in Tris-buffered saline (0.15 M NaCl, 0.1 M Tris–HCl, pH 7.5) were treated with 0.6% H_2O_2, blocked in 3% donkey serum and 0.1% Triton-X 100, and incubated with the following primary antibodies: BrdU rat monoclonal (1 : 500; Oxford Biotechnology, Oxford, UK), doublecortin (DCX) goat polyclonal (1 : 250; Santa Cruz Biotechnology, Santa Cruz, California, USA), PCNA mouse monoclonal (1 : 500; Santa Cruz Biotechnology), and V5 mouse monoclonal (1 : 1000; Invitrogen, Carlsbad, California, USA). V5 antibody was used to detect V5-His-tagged transgenic torsinA. Secondary antibodies used were donkey-derived, species-specific, and conjugated with Alexa-488 (1 : 1000; Molecular Probes, Eugene, Oregon, USA), Alexa-568 (1 : 1000; Molecular Probes), or biotin (1 : 500; Jackson Immuno Research, West Grove, Pennsylvania, USA). For immunohistochemistry, tissue was treated with avidin-biotin-peroxidase complex (1 : 100; Vector Laboratories, Burlingame, California, USA) and 3,3′-diaminobenzidine substrate kit (Vector Laboratories) to generate a black-colored product. For immunofluorescence, nuclei were counterstained with 0.5 µM To-Pro-3 dye (Molecular Probes). For all antibodies, control stainings omitting the primary antibody produced no signal.

Microscopy

Fluorescence images were obtained on a confocal scanning laser microscope (Leica TCS-NT, Bensheim, Germany) equipped with a $20 \times$ PL FLUOTAR oil objective (0.75 numeric aperture) and a pinhole setting that corresponded to a focal plane of 2 µm or less.

Quantification

All counting procedures were performed on blind-coded slides. To count BrdU-positive and PCNA-positive cells, every 6th section was sampled for a total of seven sections per hemisphere and absolute cell numbers per anatomical region of one hemisphere were obtained by multiplication with 6. To obtain total numbers for the whole brain, values were doubled. Cells within the DG, the glomerular cell layer (GLOM) of the OB, and the striatum were counted. BrdU-positive cells in the granular cell layer (GCL) of the OB were estimated with the optical fractionator method [17] using a virtual counting frame of 30×30 mm spaced in a 300×300 mm counting grid. All counting procedures and measurements of reference volumes were conducted on a light microscope (Leica, Wetzlar, Germany) equipped with a semiautomatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, Vermont, USA). Statistical analysis was performed using one-way analysis of variance comparisons between hWT, hΔGAG, and control groups (Prism 5; GraphPad Software, San Diego, California, USA). Significance threshold was assumed at P value of less than 0.05. Data are shown as mean ± standard error of the mean.

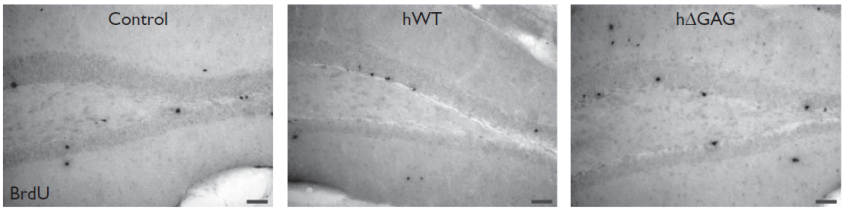
Results

Proliferation and survival of neural precursor cells are unchanged in mice transgenic for torsinA

BrdU-immunopositive cells of the neurogenic regions 4 weeks after BrdU labeling represent the survival of newly generated neuroblasts and newborn neurons. BrdU-positive cells were similarly distributed in the DG (Fig. 1) as well as in the subventricular zone – rostral migratory stream – OB pathway. Stereological quantifications of neurogenesis further showed that proliferation (PCNA) and survival (BrdU) within the DG were neither influenced by hWT nor by hΔGAG torsinA (Table 1). In addition, in the OB where neuroblasts integrate as γ-aminobutyric acidergic or dopaminergic neurons, hWT and hΔGAG torsinA exerted no effect on cell survival in the GCL and GLOM. Volumes of DG, GCL, and GLOM and densities of labeled cells were similar for controls, hWT, and hΔGAG (Table 1).

Despite changes in white matter connectivity and dopamine levels in the striatum [12], no significant expression of torsinA in the striatum was detected by immunohistochemistry (Fig. 2b) and no differences in

Fig. 1



Distribution and number of bromodeoxyuridine (BrdU) in the dentate gyrus was similar in all groups (Control, hWT, hΔGAG; see Table 1). Scale bars, 50 μm.

Table 1 Quantification of adult neurogenesis and cellular plasticity in neurogenic regions and the striatum of transgenic and control mice

	Control	hWT	hΔGAG
Neurogenic regions[a]			
Hippocampus: DG			
BrdU-positive cells	514.5 ± 67.41	593.1 ± 66.69	551.1 ± 22.39
PCNA-positive cells	634.5 ± 127.1	535.7 ± 59.23	570.9 ± 68.38
Volumes/μm^3	0.1022 ± 0.002894	0.1061 ± 0.004004	0.1011 ± 0.002594
OB: granular cell layer			
BrdU-positive cells	46500 ± 2689	49710 ± 4162	52030 ± 3958
Volumes/μm^3	1.545 ± 0.08600	1.517 ± 0.05201	1.531 ± 0.04356
OB: glomerular cell layer			
BrdU-positive cells	3979 ± 415.7	3585 ± 562.3	3979 ± 415.7
Volumes/μm^3	0.5820 ± 0.05155	0.5757 ± 0.08131	0.6240 ± 0.05153
Striatum[b]			
BrdU-positive cells	1910 ± 208.9	1621 ± 112.0	1443 ± 112.5
DCX-positive cells	19 ± 2.9	21 ± 3.5	24 ± 4.0

All values are expressed in mean ± standard error of the mean.
BrdU, bromodeoxyuridine; DCX, doublecortin; PCNA, proliferating cell nuclear antigen.
[a]Quantification of BrdU-positive cells and PCNA-positive cells revealed no effect of genotype on neuronal survival and proliferation in both neurogenic regions.
[b]No differences of newly generated BrdU-labeled cells and DCX-positive cells were detected in the striatum.

the number and the distribution of DCX-positive cells and BrdU-positive cells were detected in both types of transgenic mice (Table 1).

Transgenic human wild-type torsinA is not expressed in neuroblasts of hWT and hΔGAG animals

To determine where the murine PrP promoter drove transgene expression, we performed immunohistochemistry for the V5-His tag. In both groups, transgenic torsinA was highly present in areas of the brainstem and cerebellum as described earlier [12]. In the DG, subventricular zone, and rostral migratory stream, no expression of torsinA was observed (Fig. 2a and b). However, in the OB of hWT animals, mitral cells surrounding the GCL were immunopositive for hWT torsinA, including cellular processes tightly juxtaposed to the GLOM (Fig. 2c, d and e). These cells also presented intracellular perinuclear inclusion bodies positive for hWT torsinA (Fig. 2f). These processes and inclusions resembled affected cells of the gigantocellular reticular nucleus and the vestibular nucleus of the brainstem in this transgenic model [12]. We found that no cells were double positive for DCX and hWT torsinA in the mitral cell layer of the OB, which is consistent with the fact that newborn neuroblasts do not integrate into this cell layer. Rather, we observed a tight proximity between the processes of newborn granule and glomerular cells in the neuroblast stage and the dendrites of mitral cells containing hWT torsinA (Fig. 2g).

Discussion

Altered cellular plasticity during brain development and adulthood may lead to the underlying structural substrate for *DYT1*-associated dystonia. Here, we investigated the effect of hWT and hΔGAG torsinA on adult neurogenesis and striatal plasticity in transgenic mice. Unexpectedly, no alteration in these regions was found despite the close relation of torsinA inclusions and newly integrated neuroblasts in the OB.

Endogenous torsinA regulates neuronal development

In humans and in rodents, torsinA is weakly expressed in cells of all grey matter regions with high levels in motor areas including cerebellar Purkinje cells, dopaminergic neurons of the substantia nigra and neurons of the basal ganglia, as well as the hippocampus [18,19]. TorsinA peak expression levels coincide with the embryonic periods of neuronal process development and synapse formation and remain elevated in the hippocampus paralleling

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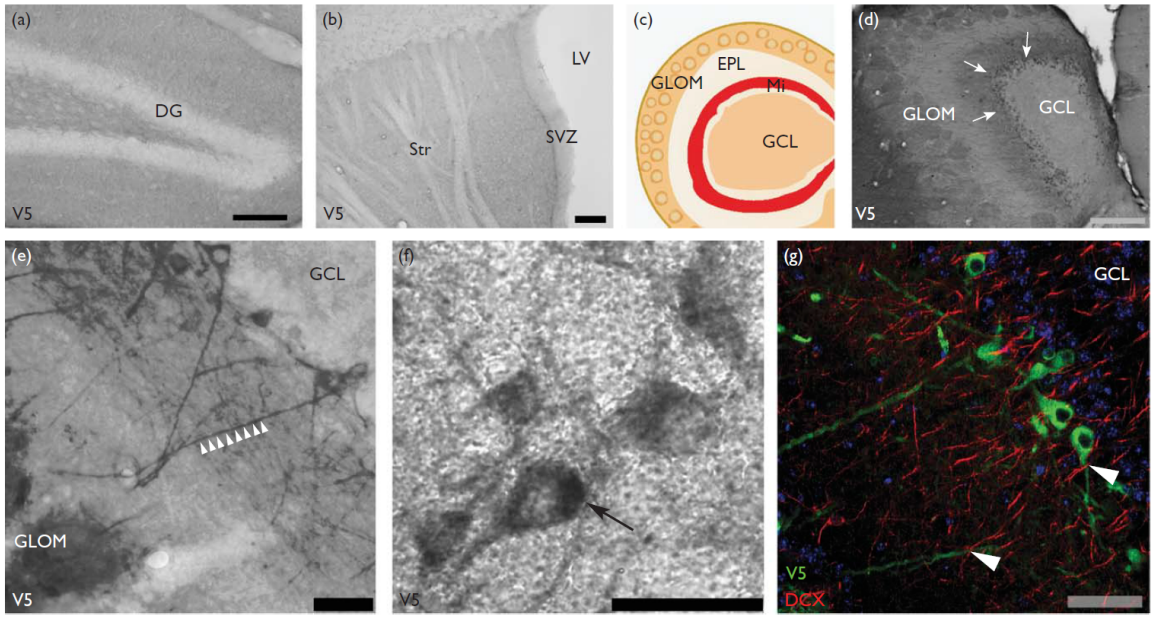
Fig. 2



Transgene expression is visualized by the V5-antibody which recognizes V5-tagged transgenic torsinA and is absent in the (a) dentate gyrus (DG) and the (b) striatum (str)/subventricular zone (SVZ), but present in hWT torsinA overexpressing animals in the perigranular cell population (d). (c) Schematic drawing of the olfactory bulb and the expression pattern of hWT torsinA. (e) V5-positive processes project radially into the glomerular cell layer (GLOM) (arrowheads). (f) Mitral cell bodies containing V5-immunopositive inclusion-like formations (arrow). (g) Doublecortin (DCX)-labeled neuroblasts (red) show no coexpression with V5 (green), but show close interaction with V5-immunopositive dendrites (arrowheads). Nuclear counterstain in blue. Scale bars, 100 μm (a, b), 1 mm (d), 25 μm (e–g). EPL, external plexiform layer; GCL, granular cell layer; LV, lateral ventricle; Mi, mitral cell layer.

postnatal neurogenesis [5,20]. Interestingly, this developmental stage overlaps with the timeframe of disease onset in human *DYT1* mutation carriers, further indicating a role for torsinA in synaptic plasticity.

TorsinA expression in transgenic mice

Both transgenic hWT and hΔGAG torsinA were expressed under the control of the PrP promoter, which is physiologically active in postmitotic mature neurons [21,22]. However, transgenic torsinA was not detected in multiple mature neuron types probably because of copy numbers or integration sites of the transgene [23]. Transgenic torsinA was found at high levels in pons, brainstem, cerebellum, and the OB. Similar expression patterns have been detected in other mice overexpressing a transgene under the PrP promoter [24]. Thus, one explanation for the lack of effects of torsinA on adult neurogenesis may be the disparity between temporal and spatial expression patterns of endogenous murine and transgenic human torsinA: while endogenous murine torsinA is expressed during development, the PrP promoter predominantly drives expression in mature neurons. In addition, early onset torsion dystonia has a penetrance of only 30% among *DYT1* mutation carriers pointing towards additional genetic or environmental factors to develop the disease [1].

Striatal pathology does not induce aberrant neuroblast migration

We hypothesized that the migration of newborn cells could be perturbated or, because of the striatal pathology, redirected toward the striatum following pathogenesis caused by expression of hWT or hΔGAG torsinA, similar to animal models with ischemic lesions. Thus, we carefully analyzed the presence of DCX-labeled cells and BrdU-labeled cells throughout the striatum. No differences were found in DCX-expressing cells and BrdU incorporation between transgenic and control mice, and we found no indication of redirected migration of neuroblasts or alterations of striatal cellular plasticity, which was further supported by the finding that transgenic torsinA was not expressed in the striatum.

TorsinA impairs neuronal function in neurogenic regions, but has no effects on survival and synaptic integration of adult neural precursor cells

As we found torsinA-related inclusion bodies in the mitral cells of the OB in close relation to the GCL and the GLOM, we expected an effect of torsinA at the integration site of newborn granule cells. Against our own anticipation, torsinA-related inclusions, however, did not affect the survival of newly born cells despite its

accumulation in the local microenvironment. Intraneuronal inclusions were also observed in a previously characterized transgenic animal model overexpressing human α-synuclein in mature granule cells of the OB [16]. In that model, however, inclusions led to an increased cell death of newly generated neurons. Therefore, we hypothesize that α-synuclein expression in newborn neurons may lead to cell-autonomous effects at their integration site. The lack of torsinA-associated cell-non-autonomous effects may be because of its lack of expression within mature newborn neurons compared with α-synuclein.

Conclusion

Transgenic forms of hWT and hΔGAG torsinA exert neither cell-autonomous nor cell-non-autonomous effects on adult neural precursor proliferation, survival, and integration. In particular, the integration of interneurons in the OB is not disturbed despite the presence of transgenic torsinA within the mitral cell population. Although the presence of striatal pathology was reported earlier [12], no redirection of newly born cells towards the striatum was observed. These results contrast to models with ischemic lesions and of synucleinopathy.

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G. Appendix

1. Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
6-OHDA	6-Hydroxydopamine
AD	Alzheimer's disease
BDNF	Brain-derived neurotrophic factor
BiP	Immunoglobulin heavy chain-binding protein
BrdU	Bromodeoxyuridine
CA3/CA1	Parts of the hippocampus ("cornu ammonis")
CMV	Cytomegalovirus
CNS	Central nervous system
DCX	Doublecortin
DG	Dentate gyrus
DOPAC	Dihydroxyphenylacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FGF-2	Fibroblast growth factor 2
GABA	γ-aminobutyric acid
GCL	Granular cell layer of the olfactory bulb
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLOM	Glomerular cell layer of the olfactory bulb
HD	Huntington's disease
HVA	Homovanillic acid
M	Molar concentration (mol/L)
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
NeuN	Neuronal nuclei
NSE	Neuron-specific enolase
OB	Olfactory bulb
PD	Parkinson's disease
PET	Positron emission tomography
PSA-NCAM	Polysialylated form of the neural cell adhesion molecule

PTD	Primary torsion dystonia
RMS	Rostral migratory stream
SGZ	Subgranular zone of the dentate gyrus
SNc	Pars compacta of the substantia nigra
SVZ	Subventricular zone of the lateral ventricles
TMS	Transcranial magnetic stimulation
VEGF	Vascular endothelial growth factor

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4. Curriculum Vitae

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Originalarbeiten

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Poster

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